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PTO/SB/05 (11-00)

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**UTILITY  
PATENT APPLICATION  
TRANSMITTAL**

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	4626US
First Inventor	Maria G. Pau et al.
Title	PRODUCTION OF VACCINES
Express Mail Label No.	EL700253940US

09/722867

11/27/00

**APPLICATION ELEMENTS**

See MPEP chapter 600 concerning utility patent application contents.

1. ☒ Fee Transmittal Form (e.g., PTO/SB/17)  
(Submit an original and a duplicate for fee processing)
  2. ☒ Applicant claims small entity status.  
See 37 CFR 1.27.
  3. ☒ Specification [Total Pages (preferred arrangement set forth below)
    - Descriptive title of the invention
    - Cross Reference to Related Applications
    - Statement Regarding Fed sponsored R & D
    - Reference to sequence listing, a table, or a computer program listing appendix
    - Background of the Invention
    - Brief Summary of the Invention
    - Brief Description of the Drawings (if filed)
    - Detailed Description
    - Claim(s)
    - Abstract of the Disclosure
  4. ☒ Drawing(s) (35 U.S.C. 113) [Total Sheets   - 5. Oath or Declaration [Total Pages   - a. ☐ Newly executed (original or copy)  
Copy from a prior application (37 CFR 1.63 (d))  
(for continuation/divisional with Box 18 completed)
  - b. ☐ **DELETION OF INVENTOR(S)**  
Signed statement attached deleting inventor(s)  
named in the prior application, see 37 CFR  
1.63(d)(2) and 1.33(b)
6. ☐ Application Data Sheet. See 37 CFR 1.76

**ADDRESS TO:**Assistant Commissioner for Patents  
Box Patent Application  
Washington, DC 20231

7. ☐ CD-ROM or CD-R in duplicate, large table or  
Computer Program (Appendix)
8. Nucleotide and/or Amino Acid Sequence Submission  
(if applicable, all necessary)
  - a. ☐ Computer Readable Form (CRF)
  - b. Specification Sequence Listing on:
    - i. ☐ CD-ROM or CD-R (2 copies); or
    - ii. ☐ paper
  - c. ☐ Statements verifying identity of above copies

**ACCOMPANYING APPLICATION PARTS**

9. ☐ Assignment Papers (cover sheet & document(s))
10. ☐ 37 CFR 3.73(b) Statement ☐ Power of  
(when there is an assignee) Attorney
11. ☐ English Translation Document (if applicable)
12. ☒ Information Disclosure ☐ Copies of IDS  
Statement (IDS)/PTO-1449 Citations
13. ☒ Preliminary Amendment
14. ☒ Return Receipt Postcard (MPEP 503)  
(Should be specifically itemized)
15. ☐ Certified Copy of Priority Document(s)  
(if foreign priority is claimed)
16. ☐ Request and Certification under 35 U.S.C. 122  
(b)(2)(B)(i). Applicant must attach form PTO/SB/35  
or its equivalent.
17. ☐ Other: \_\_\_\_\_

18. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in a preliminary amendment, or in an Application Data Sheet under 37 CFR 1.76:

☐ Continuation ☐ Divisional ☒ Continuation-in-part (CIP)of prior application No. 09/449,854

Prior application information.

Examiner \_\_\_\_\_

Group Art Unit \_\_\_\_\_

For CONTINUATION OR DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 5b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

**19. CORRESPONDENCE ADDRESS**☒ Customer Number or Bar Code Labelor ☐ Correspondence address below

Name

24247

PATENT TRADEMARK OFFICE

Address

City

State

Zip Code

Country

Telephone

Fax

Name (Print/Type)

Allen G. Turner

Registration No. (Attorney/Agent)

33,041

Signature

Date

11/27/00

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# FEE TRANSMITTAL for FY 2000

Patent fees are subject to annual revision  
Small Entity payments *must* be supported by a small entity statement,  
otherwise large entity fees must be paid. See Forms PTO/SB/09-12.  
See 37 C.F.R. §§ 1.27 and 1.28.

## Complete if Known

Application Number	
Filing Date	November 24, 2000
First Named Inventor	Maria G. Pau et al.
Examiner Name	
Group / Art Unit	
Attorney Docket No.	4626US

**TOTAL AMOUNT OF PAYMENT** (\$) 355.00

### METHOD OF PAYMENT (check one)

1. ☒ The Commissioner is hereby authorized to charge indicated fees and credit any overpayments to:

Deposit Account Number 20-1469

Deposit Account Name Trask Britt

☒ Charge Any Additional Fee Required  
Under 37 CFR §§ 1.16 and 1.17

2. ☒ Payment Enclosed:  
☒ Check ☐ Money Order ☐ Other

### FEE CALCULATION

#### 1. BASIC FILING FEE

Large Entity Code	Large Entity Fee (\$)	Small Entity Code	Small Entity Fee (\$)	Fee Description	Fee Paid
101	690	201	345	Utility filing fee	355
106	310	206	155	Design filing fee	0
107	480	207	240	Plant filing fee	0
108	690	208	345	Reissue filing fee	0
114	150	214	75	Provisional filing fee	0

**SUBTOTAL (1)** (\$) 355.00

#### 2. EXTRA CLAIM FEES

Total Claims	Extra Claims	Fee from below	Fee Paid
18	-20** = 0	9	0
3	-3** = 0	39	0
Multiple Dependent		0	0

\*\*or number previously paid, if greater. For Reissues, see below

Large Entity Code	Large Entity Fee (\$)	Small Entity Code	Small Entity Fee (\$)	Fee Description
103	18	203	9	Claims in excess of 20
102	78	202	39	Independent claims in excess of 3
104	260	204	130	Multiple dependent claim, if not paid
109	78	209	39	** Reissue independent claims over original patent
110	18	210	9	** Reissue claims in excess of 20 and over original patent

**SUBTOTAL (2)** (\$) 0.00

### FEE CALCULATION (continued)

#### 3. ADDITIONAL FEES

Large Entity Code	Large Entity Fee (\$)	Small Entity Code	Small Entity Fee (\$)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	380	216	190	Extension for reply within second month	
117	870	217	435	Extension for reply within third month	
118	1,360	218	680	Extension for reply within fourth month	
128	1,850	228	925	Extension for reply within fifth month	
119	300	219	150	Notice of Appeal	
120	300	220	150	Filing a brief in support of an appeal	
121	260	221	130	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1,210	241	605	Petition to revive - unintentional	
142	1,210	242	605	Utility issue fee (or reissue)	
143	430	243	215	Design issue fee	
144	580	244	290	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Petitions related to provisional applications	
126	240	126	240	Submission of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	
146	690	246	345	Filing a submission after final rejection (37 CFR § 1.129(a))	
149	690	249	345	For each additional invention to be examined (37 CFR § 1.129(b))	

Other fee (specify) \_\_\_\_\_

Other fee (specify) \_\_\_\_\_

\* Reduced by Basic Filing Fee Paid

**SUBTOTAL (3)** (\$) \_\_\_\_\_

### SUBMITTED BY

Name (Print/Type)	<u>Allen C. Turner</u>	Registration No (Attorney/Agent)	<u>33,041</u>	Telephone	<u>(801) 532-1922</u>
Signature	<u>[Signature]</u>	Date	<u>11/27/2000</u>		

Complete (if applicable)

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**PATENT**

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

**In re Application of:**

Maria G. Pau et al.

**Serial No.:** To be assigned

**Filed:** November 27, 2000

**For:** PRODUCTION OF VACCINES

**Examiner:** To be assigned

**Group Art Unit:** To be assigned

**Attorney Docket No.:** 4626US

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: EL700253940US

Date of Deposit with USPS: November 27, 2000

Person making Deposit: Jared Turner

**Preliminary Amendment**

Box Patent Application  
Commissioner for Patents  
Washington, D.C. 20231

Sir:

Before calculation of the filing fee, please amend the referenced application as follows:

**IN THE CLAIMS:**

Please cancel claims 2 through 20, 22 through 35, and 37 without prejudice or disclaimer.

21. (Amended) The method according to [any one of claims 1-16,]claim 1 wherein said virus is selected from the group consisting of rotavirus, coronavirus, transmissible gastroenteritis virus, flavivirus, tick-borne encephalitis virus, and yellow fever virus.

36. (Amended) A kit for determining activity of a protease in a sample, said kit comprising: at least one viral protein or virus [obtainable by a method according to any one of claims 1-25 or by a use according to any one of claims 26-30]produced by a process for producing said virus and/or viral protein, said process comprising:  
providing a cell with at least a sequence encoding at least one gene product of an adenoviral E1 gene or a functional derivative of said adenoviral E1 gene,  
providing said cell with a nucleic acid sequence encoding said desired virus and/or said desired viral proteins,  
culturing said cell in a suitable medium and allowing for expression of said virus and/or said viral proteins, and  
harvesting said virus and/or viral proteins from said suitable medium and/or said cell.

38. (Amended) The kit of claim 36 [or the use according to claim 37], wherein said protease comprises trypsin.

39. (Amended) The kit according to claim 36 [or the use according to claim 37], wherein said sample comprises culture medium.

40. A method for concentrating influenza virus under conditions capable of at least in part preserving virus infectivity, said method comprising:

obtaining a cell cleared supernatant comprising said virus from a culture of cells, and ultrafiltrating said supernatant under low shear conditions.

41. The method according to claim 40, wherein said low shear conditions are obtained with a hollow fiber.

42. (Amended) The method according to claim 40 [or claim 41], wherein said culture of cells comprise *in vitro* cultured cells.

43. (Amended) The method according to [any one of claims 40-42]claim 40, wherein said ultrafiltration is performed with filter comprising a cut off of 750 KD.

44. (Amended) The method according to [any one of claims 40-43]claim 40, wherein said concentration further comprises at least a partial removal of proteins comprising a molecular weight smaller than 750 KD.

45. Infectious influenza virus or derivatives thereof [concentrated with]produced by a method according to [any one of claims 40-44]claim 40.

Please add the following new claims:

46. The method according to claim 41, wherein said culture of cells comprise *in vitro* cultured cells.

47. The method according to claim 41, wherein said ultrafiltration is performed with filter comprising a cut off of 750 KD.

48. The method according to claim 46, wherein said ultrafiltration is performed with filter comprising a cut off of 750 KD.

49. The method according to claim 41, wherein said concentration further comprises at least a partial removal of proteins comprising a molecular weight smaller than 750 KD.

50. The method according to claim 42, wherein said concentration further comprises at least a partial removal of proteins comprising a molecular weight smaller than 750 KD.

51. The method according to claim 43, wherein said concentration further comprises at least a partial removal of proteins comprising a molecular weight smaller than 750 KD.

52. Infectious influenza virus concentrated by the method according to claim 41.

### Remarks

The application is to be amended as previously set forth. All amendments, including the cancellation of claims, are made without prejudice or disclaimer. The amendments are made to bring the application closer to United States practice, such as, by example, removing multiple claim dependencies and to remove claims redundant with the parent application.

If questions exist after consideration of the foregoing, the Office is kindly requested to contact the applicants' representative at the address or telephone number below.

Respectfully submitted,



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Date: November 27, 2000

PATENT

Attorney Docket 4626US

CERTIFICATE OF MAILING

Express Mail Label Number: EL700253940US

Date of Deposit: November 27, 2000

Person making Deposit: Jared Turner

APPLICATION FOR LETTERS PATENT

for

**PRODUCTION OF VACCINES**

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## PRODUCTION OF VACCINES

Cross-reference to related applications: This application is a continuation-in-part of U. S. patent application serial no. 09/449,854 filed on November 26, 1999, the entire contents of which are incorporated by this reference.

5        Technical Field: This invention relates generally to the development and manufacture of vaccines. In particular, the invention relates to the production of viral proteins and/or viruses, using a mammalian (*e.g.*, human) cell for the production of viruses growing in eukaryotic, especially mammalian and human, cells. The invention is useful for the production of vaccines to aid in protection against viral pathogens for vertebrates, such as mammals.

10

Background: Presently, vaccination is the most important route of dealing with viral infections. Although a number of antiviral agents are available, typically these agents have limited efficacy. Administering antibodies against a virus may be a good way of dealing with viral infections once an individual is infected (passive immunization) and typically human or  
15 humanized antibodies hold promise for dealing with a number of viral infections, but the most efficacious and safe way of dealing with virus infection presently is and probably will be prophylaxis through active immunizations. Active immunization is generally referred to as “vaccination”. Vaccines comprise at least one antigenic determinant (typically of a virus), preferably a number of different antigenic determinants of at least one virus or other pathogen,  
20 for instance, by incorporating in the vaccine at least one (viral) polypeptide or protein derived from a virus (subunit vaccines).

          Typically, vaccines include adjuvants in order to enhance the immune response. Use of adjuvants is also possible for vaccines that use whole virus (pathogen), for instance, when the virus is inactivated. Another possibility is the use of live, but attenuated, virus. A further  
25 possibility is the use of wild-type (“wt”) virus, for instance, in cases where adult individuals



are not in danger of infection, but infants are, and may be protected through maternal antibodies and the like.

Producing vaccines is not always an easy procedure. In some cases, the production of viral material is on eggs, which may lead to materials that are difficult to purify and require  
5 extensive safety measures against, for instance, contamination. Likewise, production on bacteria or yeast, which is sometimes an alternative for eggs, can require many purification and safety steps.

Production on mammalian cells would be an alternative, but the mammalian cells used thus far have required, for instance, the presence of serum and/or adherence to a solid support  
10 for growth. In the first case, again purification and safety and, for example, the requirement of protease to support the replication of some viruses becomes an issue. In the second case, high yields and ease of production become a further issue. The present invention overcomes at least a number of the problems encountered with the production systems for production of viruses and/or viral proteins for vaccine purposes of the systems of the prior art.

15

### **Description of the Invention**

The invention includes a novel human immortalized cell line for the purpose of propagating, harvesting and producing virus. PER.C6 cells (*see, e.g.*, U. S. Patent 5,994,128 to Bout et al. also deposited under No. 96022940 at the European Collection of Animal Cell  
20 Cultures at the Centre for Applied Microbiology and Research) were generated by transfection of primary human embryonic retina cells, using a plasmid that contained the adenovirus ("Ad") serotype 5 (Ad5) E1A- and E1B-coding sequences (Ad5 nucleotides 459-3510) under the control of the human phosphoglycerate kinase (PGK) promoter.

The following features make PER.C6 or a derivative thereof particularly useful as a  
25 host for virus production: it is a fully characterized human cell line, it was developed in compliance with good laboratory practices, it can be grown as a suspension culture in defined serum-free medium, devoid of any human or animal serum proteins; its growth is compatible

with roller bottles, shaker flasks, spinner flasks and bioreactors, with doubling times of about 35 hours.

### Influenza epidemiology.

Influenza viruses, members of the family of *Orthomyxoviridae*, are the causative agents of annual epidemics of acute respiratory disease. In the US alone, 50 million Americans get the flu each year. Estimated deaths worldwide (1972-1992) are 60,000 (CDC statistics). There have been 3 major cases of pandemics of influenza, namely in 1918 (Spanish flu, est. 40 million deaths), in 1957 (Asian flu, est. 1 million deaths), and in 1968 (Hong-Kong flu, est. 700.00 deaths).

Infections with influenza viruses are associated with a broad spectrum of illnesses and complications that result in substantial worldwide morbidity and mortality, especially in older people and patients with chronic illness. Vaccination against influenza is most effective in preventing the often fatal complications associated with this infection (Murphy, B.R and R.G. Webster 1996). The production of influenza virus on the diploid human cell line MRC-5 has been reported (Herrero-Euribe L et al. 1983). However, the titers of influenza virus were prohibitively low.

### Strains of Influenza virus

Present day flu vaccines contain purified hemagglutinin and neuraminidase of Influenza virus A and B. The 3 viruses that represent epidemiological important strains are Influenza A (H1N1), Influenza A (H3N2) and Influenza B. The division into A and B types is based on antigenic differences between their nucleoprotein (NP) and matrix (M) protein antigen. The Influenza A virus is further subdivided into subtypes based on the antigenic composition (sequence) of hemagglutinin (H1-H15) and neuraminidase (N1-N9) molecules. Representatives of each of these subtypes have been isolated from aquatic birds, which, probably, are the primordial reservoir of all influenza viruses for avian and mammalian species. Transmission has been shown between pigs and humans and, recently (H5N1), between birds and humans.

### Influenza vaccines

Three types of inactivated influenza vaccine are currently used in the world: whole virus, split product, and surface antigen or “subunit” vaccines. These vaccines all contain the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), of the influenza virus strains that are expected to circulate in the human population in the upcoming season. These strains, which are incorporated into the vaccine, are grown in embryonated hens' eggs, and the viral particles are subsequently purified before further processing.

The need for the yearly adjustment of influenza vaccines is due to antigen variation caused by processes known as “antigenic drift” and “antigenic shift”.

“Antigenic drift” occurs by the accumulation of a series of point mutations in either the H or N protein of a virus resulting in amino acid substitutions. These substitutions prevent the binding of neutralizing antibodies, induced by previous infection, and the new variant can infect the host.

“Antigenic shift” is the appearance of a new subtype by genetic re-assortment between animal and human Influenza A viruses. The pandemic strains of 1957 (H2N2) and 1968 (H3N2) are examples of re-assorted viruses by which avian H and or N genes were introduced in circulating human viruses, that subsequently spread among the human population.

Based on the epidemiological surveys by over one hundred National Influenza Centres worldwide, the World Health Organization (WHO) yearly recommends the composition of the influenza vaccine, usually in February for the northern hemisphere, and in September for the southern hemisphere. This practice limits the time window for production and standardization of the vaccine to a maximum of 9 months.

If an urgent demand arises for many doses of vaccine, for example when a novel subtype of Influenza A virus arises by antigenic shift or antigenic drift, limited availability of eggs may hamper the rapid production of vaccine. Further disadvantages of this production system are the lack of flexibility, the risk of the presence of toxins, and the risks of adventitious viruses, particularly retroviruses, and concerns about sterility. These

disadvantages present a serious problem in today's practice of influenza vaccine production on embryonated hens' eggs.

Therefore, the use of a cell culture system for influenza vaccine production would be an attractive alternative. Influenza viruses can be grown on a number of primary cells, including monkey kidney, calf kidney, hamster kidney and chicken kidney. Yet, their use for vaccine production is impractical, due to the need to re-establish cultures from these primary cells for each preparation of a vaccine. Therefore, the use of continuous immortalized cell lines for influenza vaccine production is an attractive alternative.

The use of culture systems was facilitated by the realization that the proteolytic cleavage of HA into its two subunits (HA1 and HA2) is required for influenza virus infectivity, and can be obtained by adding trypsin. Including trypsin permits replication and plaque formation in Madin-Darby canine kidney (MDCK) cells (Tobita et al. 1975).

The MDCK cell line was recently shown to support the growth of influenza virus for vaccine production (Brand et al. 1996 and 1997, Palache et al. 1997). The use of trypsin requires growth of the MDCK cells in serum-free tissue culture medium (MDCK-SF1). However, MDCK cells are currently not approved as a substrate for production of influenza virus.

Importantly, any non-human system for producing influenza vaccines has an inherent drawback, known as 'adaptation'. Human Influenza A and B viruses both carry mutations in HA, due to adaptation in embryonated hens' eggs. These mutations result in altered antigenicity (Newman et al. 1993, Williams and Robertson 1993, Robertson et al. 1994, Gubareva et al. 1994, Schild et al. 1993, Robertson et al. 1987, Kodihalli et al. 1995). In humans, immunization with vaccines containing HA bearing an egg-adaptation mutation induces less neutralizing antibody to virus than a non-egg adapted HA (Newman et al. 1993).

Human influenza viruses propagated in canine cells such as MDCK cells also show adaptation, albeit to a lesser extent. Such viruses resemble the original human isolates more closely than egg-derived viruses (Robertson et al. 1990).

Furthermore, evidence exists that host-specific changes in NA and host-specific phosphorylation patterns of NA can affect the replication of Influenza viruses (Schulman and Palese 1977; Sugiara and Ueda 1980; Kistner et al. 1976).

Therefore, it would clearly be advantageous to avoid adaptation or other host-induced changes of influenza virus, possibly resulting in a more homogeneous population of viruses, rendering the ultimate vaccine more effective.

The present invention provides human cells used as a substrate for the production of high titers of influenza virus, suitable for the development of vaccines.

#### Rotavirus and Vaccines Therefor

Rotaviruses are the most important cause of severe dehydrating gastroenteritis in young children worldwide. In developing countries, infections with rotaviruses reportedly lead to over 800,000 deaths annually. In the United States alone, estimated costs of health care due to rotavirus infections exceed 1 billion US dollars per year.

Rotaviruses, members of the family of *Reoviridae*, are double stranded RNA viruses consisting of 11 RNA segments, each coding for a structural or non-structural viral protein (VP). This inner core of the virus comprises four VP's: VP1, 2, 3 and 6. These VP determine the three main antigenic properties of HRV—group, subgroup, and serotype. Seven antigenically distinct groups (denominated A through G) have been identified, that are encoded by the VP6. Infection with human rotavirus (HRV) is predominantly caused by group A rotaviruses, with serotypes 1-4 accounting for 95% of clinical illness. Natural disease protection is serotype specific. Group A is further classified into subgroups I and II.

The double layer outer shell forming the viral capsid consists of two viral proteins, VP4 and VP7, that are the neutralization antigens involved in protective immunity and that determine the serotype, although the VP4 plays a minor role in serotype determination. During co-infection with different serotypes, the segmented genomes readily undergo genetic reassorting, a property that has been used to create a vaccine (Marsha et al. 1999).

Given the worldwide prevalence of rotavirus associated infant morbidity and mortality, large scale vaccination against rotavirus is considered the most effective way to combat this virus. The goal of vaccination would not be to prevent the disease but to reduce its morbidity, especially during the first few years of life.

5        The only effective vaccine available at present is a live, attenuated, orally delivered vaccine based on the re-assortment of RNA segments of human rotaviruses, encoding the VP7's of serotypes 1, 2 and 4 in a Rhesus rotavirus supplying the attenuated background together with the VP7 of serotype 3. Vaccination with this human / rhesus reassortant tetravalent vaccine (RRV-TV), although highly effective in preventing severe gastroenteritis,  
10 is associated with intussusception, a bowel obstruction disease. For that reason, this vaccine is no longer in use.

Means and methods are disclosed herein for producing a virus and/or viral protein in a cell, preferably using a defined synthetic medium, and for purifying the virus and/or components thereof from the cell and/or culture medium. Pharmaceutical compositions  
15 containing virus or its components and methods for manufacturing and recovering and/or purifying them are provided.

#### **Brief Description of the Figures**

FIG. 1 is a graph depicting percentage of infected cells (positive cells) viewed  
20 microscopically after immunofluorescence assay versus percentage of dead cells measured via FACS after propidium iodide staining, at multiplicities of infection (moi's) of  $10^{-3}$  and  $10^{-4}$ . Poor viability of the cells from samples derived from infection at moi  $10^{-3}$  did not give rise to reliable data.

FIG. 2 are two graphs depicting percentage of infected cells viewed microscopically  
25 after immunofluorescence assay. Samples derived from infection at moi 10 and 1, at 48h post infection are not shown, because of full CPE.

FIG. 3 is a graph depicting kinetics of virus propagation measured in hemagglutinating units (HAU) from day 1 through day 6 after infection.

FIG. 4 consists of two graphs depicting percentage of infected cells (positive cells) viewed microscopically after immunofluorescence assay.

5        FIG. 5 consists of two graphs depicting kinetics of virus propagation measured in HAU from day 1 through day 6 after infection.

FIG. 6 consists of two graphs depicting percentage of infected cells (positive cells) viewed microscopically after immunofluorescence assay.

FIG. 7 consists of two graphs depicting kinetics of virus propagation measured in HAU  
10    from day 2 through day 6 after infection.

FIG. 8 consists of portions A and B depicting expression of Sia2-3Gal and Sia2-6Gal linkages on cell surface receptors present on Chinese Hamster Ovary (CHO) cells, PER.C6 cells and MDCK cells. Portion A is a schematic representation of the interaction of the Sambuca nigra agglutinin (SNA) lectin that specifically recognizes Sia2-6Gal linkages and the  
15    Maackia amurensis agglutinin (MAA) lectin that specifically recognizes Sia2-3Gal linkages. The schematic interaction with the FITC labelled anti-DIG antibody recognizing the DIG labelled lectin bound to the oligosaccharide chain on the cell surface protein is also depicted. Portion B depicts FACS analysis of cells incubated with DIG-labeled lectins. Lectins attached to the cells were detected with FITC-labeled anti-DIG antibody using procedures known to  
20    persons skilled in the art. Cell number counts are plotted against the fluorescence intensity of lectin-stained cells (gray) as compared with cells that were incubated only with the FITC-anti-DIG antibody (open). The upper panels of Portion B show the shift in the FACS analysis obtained by using the SNA lectin, while the lower panels of Portion B show the shift in the FACS analysis obtained by using the MAA lectin.

25        FIG. 9 consists of Portions A, B, and C, depicting infection with A/Sydney/5/97 on PER.C6. (A) Effect of trypsin-EDTA on HAU titers. (B) HA concentration in  $\mu\text{g/ml}$  and (C)

virus infectivity titers in pfu's/ml as measured in crude viral supernatants, 96 hours post infection.

FIG. 10 consists of Portions A, B, and C depicting infection with B/Harbin/7/94 on PER.C6. (A) Effect of different concentrations of trypsin-EDTA present during and after virus infection on growth kinetics. (B) HAU titers per 50  $\mu$ l and (C) virus infectivity titers in pfu/ml.

FIG. 11 consists of Portions A and B which depict infection with X-127 using an moi of  $10^{-3}$  on PER.C6. (A) Effect of trypsin-EDTA on HAU given in HAU/50  $\mu$ l and (B) virus infectivity titers in pfu/ml for 5 days after infection.

FIG. 12 consists of Portions A and B depicting infection with X-127 using an moi of  $10^{-4}$  on PER.C6. (A) Effect of trypsin-EDTA on HAU given in HAU/50  $\mu$ l and (B) virus infectivity titers in pfu/ml during 5 days after infection.

FIG. 13 consists of Portions A and B depicting effect of trypsin-EDTA on (A) PER.C6 cells viability and (B) biological activity of the virus. Cell viability was measured after trypan-blue staining. HAU titers were measured as described and given per 50  $\mu$ l.

FIG. 14 consists of Portions A and B depicting effect of trypsin-EDTA on virus infectivity titers and HA protein content after influenza infection of PER.C6 cells with A/Sydney/5/97. (A) The infectivity assay was carried out by inoculating, in quadruplicate, MDCK cells with a total of 100  $\mu$ l of 10-fold serially diluted virus containing supernatants, in serum free medium with trypsin-EDTA (4  $\mu$ g/ml). After seven days, supernatant of these cultures were tested for HA activity. The infectious virus titers were calculated according to the method of Spearman-Kärber (1931). (B) Western blot analysis of the A/Sydney/5/97 HA protein. Harvesting of viral proteins were carried out by disruption and denaturation of proteins using an SDS containing lysis buffer. The electrophoretic run was performed on a 10% SDS/PAGE gel under reducing conditions. Separated proteins were probed with the specific anti-A/Sydney-HA antisera. Increasing amounts of the positive control A/Sydney HA antigen (left 4 lanes) and 10  $\mu$ l of PER.C6 cells supernatants of the indicated trypsin incubated samples (right 5 lanes) were loaded.



FIG. 15 consists of an upper and lower portion and depict PER.C6 cells viability, glucose concentration, and growth kinetics of A/Sydney/5/97 in a hollow fiber perfusion system..

FIG. 16 consists of a right and left portion, and depicts the characterization and quantification of Influenza Virus A/Sydney/5/97 propagated on PER.C6 in a hollow fiber perfusion system. SDS-PAGE and Western blots were done as described in legend to FIG. 14 for the Sheep anti-A/Sydney-HA antibody. The monoclonal antibody anti HA-tag (HA probe (F7), mouse monoclonal, (Santa Cruz) was used in 1:1000 dilution. As a second antibody a goat anti mouse-HRP conjugated antibody (Biorad), in 1:7500 dilution was used.

FIG. 17 consists of a right and left graph, and depicts PER.C6 cells viability (left panel) and glucose concentration (right panel) in a 12 liter bioreactor up to 92 h after viral infection using A/Sydney/5/97 virus.

FIG. 18 is a graph depicting the infection of PER.C6 with A/Sydney/5/97 in a 10 liter cell suspension in a 12 liter bioreactor. Kinetics of virus replication as measured by immunofluorescence assay are given in percentages of positively stained cells.

FIG. 19 is a bar graph depicting infection of PER.C6 cells with A/Sydney/5/97 in a 10 liter cell suspension in a 12 liter bioreactor. Kinetic of virus replication as measured by Hemagglutination assay are given in HAU's during several days after viral infection. The bar depicted with an asterisk is the number of HAU's obtained after Powerfuge™ clarification as described in the text.

FIG. 20 is a Western blot following infection of PER.C6 with A/Sydney/5/97 virus in a 10 liter cell suspension in a 12 liter bioreactor. Shown is the characterization and quantification of the Influenza virus A/Sydney/5/97 HA polypeptide. SDS/PAGE and Western blot were done as described with respect to FIG. 14. The different subunits (HA1 and HA2) and the non-cleaved HA0 proteins are depicted by arrow heads. The HA obtained from NIBSC served as a positive control.

FIG. 21 shows the determination of HAU's and pfu/ml after infection of PER.C6 with A/Sydney/5/97 in a 10 liter cell suspension in a 12 liter bioreactor. The infection was followed by Down Stream Processing (DSP). The recovery of viral yields after hollow fiber ultra-filtration (20 fold concentration) is also shown.

5        FIG. 22 consists of four graphs depicting infection of PER.C6 with A/Sydney/5/97 in a 2 liter cell suspension in a 3 liter bioreactor. PER.C6 cells viability (upper left), glucose concentration (upper right) and growth kinetics of the virus in the percentage of positively staining cells (lower left), and HAU's (lower right) are given.

FIG. 23 consists of four graphs depicting infection of PER.C6 with A/Beijing/262/95  
10    in a 2 liter cell suspension in a 3 liter bioreactor. PER.C6 cells viability (upper left), glucose concentration (upper right) and growth kinetics of the virus in the percentage of positively staining cells (lower left), and HAU's (lower right) are given.

FIG. 24 consists of four graphs depicting infection of PER.C6 with B/Harbin/7/94 in a 2 liter cell suspension in a 3 liter bioreactor. PER.C6 cells viability (upper left), glucose  
15    concentration (upper right) and growth kinetics of the virus in the percentage of positively staining cells (lower left), and HAU's (lower right) are given.

FIG. 25 is a Western blot analysis of uncleaved A/Sydney/5/97 HA0 protein. Positive staining proteins are detected after incubation with the specific anti-A/Sydney antisera obtained from NIBSC and described as in the legend of FIG. 14 and in the text.

20        FIG. 26A is a Western blot analysis of A/Sydney/5/97 derived HA0 protein digested with trypsin. Proteins are detected after incubation with the specific anti-A/Sydney antisera. On the left a standard cleaved A/Sydney HA, on the right HA0 treated with increasing amount of trypsin.

FIG. 26B is a determination of trypsin activity in the culture supernatant of an  
25    Influenza B/Harbin production run, using HA0 of Influenza A/Sydney/5/97 as substrate. Western blot analysis of HA0 cleavage products HA1 and HA2 as visualized by anti Influenza A/Sydney/5/97 HA specific antisera mentioned in legend to FIG. 14.

FIG. 27 is a Western blot analysis of A/Sydney HA0 digested with N-glycosydase F. Proteins are detected after incubation with the specific anti-A/Sydney antisera. The protein band depicted with an asterisk is the de-glycosylated product.

FIG. 28 is a Western blot analysis of A/Sydney/5/97 HA after Accutase digestion. Proteins are detected after incubation with the specific polyclonal anti-A/Sydney-HA antisera. On the left, HA0 before and after trypsin treatment, on the right HA0 digested with decreasing amount of Accutase.

FIG. 29 consists of five portions (A through E) and depicts electron micrographs of Influenza A/Sydney/5/97. (A) PER.C6 cells 72 hrs post infection. (B and C) Negative staining on virus derived from infected PER.C6. (D and E) Negative staining of sucrose purified material.

FIG. 30A identifies different Influenza A and B strains tested on PER.C6 cells.

FIG. 30B is a bar graph depicting infectivity titers of three depicted A- and B-type influenza viruses derived from infected PER.C6 cells.

FIG. 31 consists of five bar graphs (A through E) depicting immunofluorescence of PER.C6 and Vero cells infected with viruses other than influenza. (A) Positively staining cells upon infection with Measles virus. (B) Positively staining cells upon infection of Vero cells with HSV-1 virus. (C) Positively staining cells upon infection of Vero cells with HSV-2 virus. (D) Positively staining cells upon infection of PER.C6 cells with HSV-1 virus. (E) Positively staining cells upon infection of PER.C6 cells with HSV-2 virus.

FIG. 32 consists of upper, middle and lower portions, depicting infectivity titers determined after propagation of measles virus (middle panel), HSV-1 (bottom panel) and HSV-2 (top panel) virus on PER.C6 cells.

FIG. 33 consists of an upper and lower panels, and depicts replication of rotavirus after infection of PER.C6 (top panel) and Vero (bottom panel) cells with different moi's as measured by ELISA in crude supernatants.

### Detailed Description of The Invention

The invention provides a method for producing a virus and/or viral proteins other than adenovirus or adenoviral proteins for use as a vaccine comprising providing a cell with at least a sequence encoding at least one gene product of the E1 gene or a functional derivative thereof  
5 of an adenovirus, providing the cell with a nucleic acid encoding the virus or the viral proteins, culturing the cell in a suitable medium and allowing for propagation of the virus or expression of the viral proteins and harvesting the virus and/or viral proteins from the medium and/or the cell.

Heretofore, few, if any (human) cells have been found that were suitable to produce  
10 viruses and/or viral proteins for use as vaccines in any reproducible and scalable manner and/or in sufficiently high yields and/or easily purifiable. We have now found that cells, having adenoviral E1 sequences (preferably in their genome), are capable of sustaining the propagation of viruses in significant amounts.

A preferred cell according to the invention is derived from a human primary cell,  
15 preferably a cell which is immortalized by a gene product of the E1 gene. In order to be able to grow a primary cell, it, of course, needs to be immortalized. A good example of such a cell is one derived from a human embryonic retinoblast.

In cells according to the invention, it is important that the E1 gene sequences are not lost during the cell cycle. It is therefore preferred that the sequence encoding at least one gene  
20 product of the E1 gene is present in the genome of the (human) cell.

For safety reasons, care is best taken to avoid unnecessary adenoviral sequences in the cells. It is thus another embodiment of the invention to provide cells that do not produce adenoviral structural proteins. However, in order to achieve large scale (continuous) virus production through cell culture, it is preferred to have cells capable of growing without  
25 needing anchorage. Preferred cells according to the invention have that capability. To have a clean and relatively safe production system from which it is easy to recover and, if desired, to purify the virus, it is preferred to have a method according to the invention, wherein the human

cell comprises no other adenoviral sequences. The most preferred cell for the methods and uses of the invention is the previously identified PER.C6 cell, or a derivative thereof.

Thus, the invention provides a method of using a cell according to the invention, wherein the cell further comprises a sequence encoding E2A or a functional derivative or  
5 analogue or fragment thereof, preferably a cell wherein the sequence encoding E2A or a functional derivative or analogue or fragment thereof is present in the genome of the human cell, and, most preferably, a cell wherein the E2A encoding sequence encodes a temperature sensitive (ts) mutant E2A.

Furthermore, as previously stated, the invention also provides a method wherein the  
10 (human) cell is capable of growing in suspension.

The invention also provides a method wherein the human cell can be cultured in the absence of serum. A cell according to the invention, in particular PER.C6, preferably has the additional advantage that it can be cultured in the absence of serum or serum components. Thus isolation is easy, safety is enhanced, and the system has good reliability (synthetic media  
15 are the best for reproducibility). The human cells of the invention, and in particular those based on primary cells, particularly ones based on HER cells, are capable of normal (for humans) post- and peri-translational modifications and assembly. This means that they are very suitable for preparing viral proteins and viruses for use in vaccines.

Thus, the invention provides a method wherein the virus and/or the viral proteins  
20 comprise a protein that undergoes post-translational and/or peritranslational modification, such as glycosylation.

A good example of a viral vaccine that has been cumbersome to produce in any reliable manner is influenza vaccine. The invention provides a method wherein the viral proteins comprise at least one of an influenza virus neuraminidase and/or a hemagglutinin. Other viral  
25 proteins (subunits) and viruses (wt to be inactivated) or attenuated viruses that may be produced in the methods according to the invention include enterovirus, such as rhinovirus, aphthovirus, or poliomyelitis virus, herpes virus, such as herpes simplex virus, pseudorabies

virus or bovine herpes virus, orthomyxovirus, such as influenza virus, a paramyxovirus, such as Newcastle disease virus, respiratory syncytio virus, mumps virus or a measles virus, retrovirus, such as human immunodeficiency virus or a parvovirus or a papovavirus, rotavirus or a coronavirus, such as transmissible gastroenteritis virus or a flavivirus, such as tick-borne encephalitis virus or yellow fever virus, a togavirus, such as rubella virus or Eastern-, Western-, or Venezuelan equine encephalomyelitis virus, a hepatitis-causing virus, such as hepatitis A or hepatitis B virus, a pestivirus, such as hog cholera virus or a rhabdovirus, such as rabies virus, a Bunyaviridae virus, such as Hantavirus.

In one embodiment, a cell of the invention is useful in the generation of an influenza virus strain that does not grow very efficiently on embryonal eggs.

The invention also includes the use of a human cell having a sequence encoding at least one adenoviral E1 protein or a functional derivative, homolog or fragment thereof in its genome, which cell does not produce structural adenoviral proteins for the production of a virus, or at least one viral protein for use in a vaccine. Of course, for such a use, the cells preferred in the methods according to the invention are also preferred. The invention also provides the products resulting from the methods and uses according to the invention, especially viral proteins and viruses obtainable according to those uses and/or methods, especially when brought in a pharmaceutical composition comprising suitable excipients and in some formats (inactivated viruses, subunits) adjuvants. Dosage and ways of administration can be sorted out through normal clinical testing in as far as they are not yet available through the already registered vaccines.

Thus, the invention also provides a virus or a viral protein for use in a vaccine obtainable by a method or by a use according to the invention, the virus or the viral being free of any non-human mammalian proteinaceous material and a pharmaceutical formulation comprising such a virus and/or viral protein.

The invention further provides a human cell having a sequence encoding at least one E1 protein of an adenovirus or a functional derivative, homolog or fragment thereof in its

genome, which cell does not produce structural adenoviral proteins and having a nucleic acid encoding a virus or at least one non-adenoviral viral protein. This cell can be used in a method according to the invention.

In a preferred embodiment, the invention provides influenza virus obtainable by a method according to the invention or by a use according to the invention. In another embodiment, the invention provides influenza vaccines obtainable by a method according to the invention or by a use according to the invention.

In another aspect, the invention provides a kit for determining activity of a protease in a sample comprising at least one viral protein or virus obtainable by a method or a use of the invention the virus or the viral protein being free of any non-human mammalian proteinaceous material. This aspect of the invention is useful particularly for determining protease activity in culture medium. Culture medium is noted for being a difficult context for determining activity of a protease. However, using a viral protein or a virus of the invention as a target for the protease it is possible to accurately determine activity of the protease also in culture medium. In a preferred embodiment, therefore, the protease activity in a sample comprising culture medium. In a preferred embodiment, the protease comprises trypsin. In a preferred embodiment, the viral protein comprises HA0.

In yet another aspect, the invention provides a method for concentrating influenza virus under conditions capable of, at least in part, preserving virus infectivity, comprising obtaining a cell cleared supernatant comprising the virus from a culture of cells, and ultra-filtrating the supernatant under low shear conditions. Influenza virus preparations harvested from embryonal eggs typically need to be purified for the preparation of a vaccine. Purification typically entails at least one concentration step of the virus.

Current technologies for the concentration of influenza virus from such relatively crude preparations of influenza virus are cumbersome. Using a method of concentration of the invention, it is possible to concentrate influenza virus preparations under conditions that maintain at least in part infectivity of the virus. Preferably, virus is concentrated that is or can

be made infectious. "Can be made infectious", as used herein, means the generation of infectious virus through cleavage of HA0.

In a preferred embodiment, the concentration is performed using a hollow fiber. A hollow fiber is particularly suited to concentrate under low shear conditions.

5 In a preferred embodiment, the culture of cells comprise *in vitro* cultured cells. Particularly suited for concentration using a method of the invention is supernatant from *in vitro* cultured cells. Particularly when the supernatant comprise serum free culture medium. In a preferred embodiment, the ultra-filtration is performed with filter allowing single proteins to pass while retaining virus. Preferably, the filter comprises a cut off of 500 KD. More  
10 preferably, the a filter comprises a cut of 750 KD. In a particularly preferred embodiment, the concentration further comprises at least a partial removal of proteins comprising a molecular weight smaller than 500 KD and more preferably smaller than 750 KD. Preferably, the purification is achieved using a mentioned filter.

In yet another aspect, the invention provides infectious influenza virus or derivatives  
15 thereof concentrated with a method of the invention. A derivative of an infectious influenza virus of the invention typically is a virus, virus particle, or viral protein or part thereof that can be used for immunization purposes. Typically, this entails a virus infectivity inactivation step.

To further illustrate the invention, the following examples are provided, which are not  
20 intended to limit the scope of the invention.

## EXAMPLES

### Example 1

#### Materials and Methods

#### 25 PER.C6 and MDCK Cell culture

MDCK cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies Breda, NL) containing 10% heat inactivated fetal bovine serum and 1x L-



Glutamine (Gibco-BRL), at 37°C and 10% CO<sub>2</sub>. Suspension cultures of PER.C6 were cultured in ExCell 525 (JRH Biosciences) supplemented with 1x L-Glutamine, at 37°C and 10% CO<sub>2</sub>, in stationary cultures in 6 well dishes (Greiner) or in 490 cm<sup>2</sup> tissue culture roller bottles (Corning Costar Corp.) during continuous rotation at 1 rpm.

5 Immunofluorescence test

Direct immunofluorescence assays for the detection of Influenza virus infection were carried out using the IMAGEN<sup>TM</sup> Influenza Virus A and B kit (Dako) according to the standard protocol of the supplier. Samples were viewed microscopically using epifluorescence illumination. Infected cells were characterised by a bright apple-green fluorescence.

10 Propidium Iodide staining

Cell pellets were resuspended in 300 µl of cold PBS/0.5% BSA + 5 µl of propidium iodide (concentration 50 µg/ml) in PBS/FCS/azide solution known to persons skilled in the art. Viable and dead cells were then detected via flow cytofluorometric analysis.

Hemagglutination assay

15 In general, hemagglutination assays for Influenza virus titers were performed according to methods known to persons skilled in the art. Here, 50 µl of a two-fold diluted virus solution in PBS was added to 25 µl PBS and 25 µl of a 1% suspension of turkey erythrocytes (Biotrading Benelux B.V.) in PBS and incubated in 96 well microtiter plates at 4°C for 1 h. The hemagglutination pattern was examined and scored, and expressed as hemagglutinating  
20 units (HAU's). The number of HAU's corresponded to the reciprocal value of the highest virus dilution that showed complete hemagglutination.

Western blot analysis of the Influenza HA protein.

In general, obtained influenza viruses were disrupted in a Laemmli buffer according to methods known in the art and different volumes of obtained protein mixtures were separated  
25 using 10% SDS/PAGE gels. In brief, blots were blocked for 30 min at room temperature with block solution (5% non fat dry milk powder (Biorad) in TBST supplemented with 1% rabbit serum (Rockland), followed by 3 washes with TBST. Then, the blots were incubated with the

anti A/Sydney/5/97 HA antiserum (98/768 NIBSC) diluted 1/500 in 1%BSA/TBST with 5% rabbit serum (Rockland) O/N at room temperature. Again, the blots were washed 8 times with TBST. Finally, the blots were incubated with the rabbit anti sheep antiserum (HRP labelled, Rockland) 1/6000 diluted in block solution for 1 h at room temperature. After 8 washes with  
5 TBST, the protein-conjugate complex was visualised with ECL (Amersham Pharmacia Biotech), and films (Hyperfilm, Amersham Life Science) were exposed. The antisera were obtained from the NIBSC (UK) and applied in dilutions recommended by the NIBSC.

#### Single Radial Immunodiffusion (SRID) assay

The concentration of hemagglutinin in supernatants, derived from influenza virus  
10 infected-PER.C6 cells, was determined by the single radial immunodiffusion (SRID) test as previously described (Wood et al. 1977). The assay was performed using standard NIBSC (UK) antigens and antisera reagents.

#### Plaque assay

A total of 1 ml of 10-fold serially diluted viral supernatants were inoculated on MDCK  
15 cells which were grown until 95% confluence in 6-well plates. After 1 h at 35°C, the cells were washed twice with PBS and overloaded with 3 ml of agarose mix (1.2 ml 2.5% agarose, 1.5 ml 2x MEM, 30 ml 200 mM L-Glutamine, 24 ml trypsin-EDTA, 250 ml PBS). The cells were then incubated in a humid, 10% CO<sub>2</sub> atmosphere at 35°C for approximately 3 days and viral plaques were visually scored.

#### 20 Virus infectivity assay (TCID<sub>50</sub>)

Titration of infectious virus was performed on MDCK cells. In brief, cells were seeded in 96 well plates at a density of 4x10<sup>4</sup> cells/well in DMEM supplemented with 2mM L-Glutamine. Twenty-four hours later, cells were infected with 100 µl of ten fold serially diluted culture supernatants, in quadruplicate, in medium containing Trypsin-EDTA at the  
25 concentration of 4 mg/ml. Two hours after infection, cell monolayers were washed two times in PBS and incubated in medium containing trypsin for 7 days, at 35°C. Supernatants from

these cultures were then tested in an HA assay. TCID<sub>50</sub> titers were calculated according to the method of Karber (1931).

#### b-propiolactone Influenza virus inactivation

For inactivation of the viruses to obtain whole-inactivated virus for the generation of vaccines derived from PER.C6, a mutation protocol known to persons skilled in the art was performed using b-propiolactone. b-propiolactone is a very effective agent widely used for the inactivation of viruses and well known in the art for its mutating effects. It modifies nucleic acid bases of the viral genome and the host cell genome and blocks replication thereafter. Following an established protocol used to prepare the whole inactivated influenza vaccine prepared on embryonated eggs, the amount of virus corresponding to approximately 400 mg of HA per strain was inactivated and used for the final vaccine formulation. Briefly, one volume of 0.3 M sodium phosphate buffer was added to 9 volumes of influenza virus preparation. Inactivation of the viruses was carried out adding one volume of 10% of b-propiolactone (Newall Design, UK) to 100 volumes of phosphate- buffered virus preparation and incubated at 20°C for 24 h. Inactivation of the viruses was checked by plaque assay and no plaques were detected for any of the inactivated batches (data not shown).

### **Example 2A**

#### **PER.C6 Cell Banking and Pre-culture**

Cell line PER.C6, or derivatives thereof were used. Cell lines were banked by a two tier cell bank system. The selected cell line was banked in a research master cell bank (rMCB) which was stored in different locations. From this rMCB research, working cell banks (rWCB) were prepared as follows: an ampoule of the rMCB was thawed, and cells were propagated until enough cells are present to freeze the cells by using dry ice. Up to 500 ampoules containing 1 ml (1-2x10<sup>6</sup> cells/ml) of rWCB were stored in the vapour phase of a liquid N<sub>2</sub> freezer.

One ampoule containing  $5 \times 10^6$  PER.C6 cells of the WCB was thawed in a water bath at  $37^\circ\text{C}$ . Cells were rapidly transferred into a 50 ml tube and resuspended by adding 9 ml of the suspension medium ExCell 525 (JRH Biosciences) supplemented with 1 x L-Glutamine. After 3 min of centrifugation at 1000 rpm in a tabletop centrifuge, cells were resuspended in a  
5 final concentration of  $3 \times 10^5$  cells/ml and cultured in a T80 tissue culture flask, at  $37^\circ\text{C}$ , 10%  $\text{CO}_2$ . Two to three days later, cells were seeded into 490  $\text{cm}^2$  tissue culture roller bottles (Corning Costar Corp.), with a density of  $3 \times 10^5$  per ml and cultured in continuous rotation at 1 rpm.

### Example 2B

#### PER.C6 Cells as Permissive Cell Line for Influenza A Virus

PER.C6 as a human cell was not known for its ability to sustain influenza virus infection and replication. It was therefore determined whether PER.C6 cells are permissive for influenza virus infection in comparison with the dog cell line MDCK, that served as a positive  
15 control.

On the day before infection,  $2 \times 10^5$  MDCK cells per well were seeded in 6-well plates. Twenty-four hours later,  $4 \times 10^5$  seeded PER.C6 and the MDCK cells per well were infected with the H1N1 strain A/Puerto Rico/8/34 (titer  $3.6 \times 10^7$  pfu/ml), (obtained from Dr. E. Claas, Leiden University Medical Center, The Netherlands). Infection was performed at various  
20 multiplicities of infection (moi's) ranging from 0.1 to 10 pfu/cell. After about 2 hours of incubation at  $37^\circ\text{C}$ , the inoculum was removed and replaced by fresh culture medium. A direct immunofluorescence assay for the detection of influenza virus infection was performed 24 and 48 h post infection. The experiment showed permissiveness of PER.C6 for influenza infection, with percentages of positive cells moi-dependent and comparable with MDCK (FIG. 1).

### Example 3

#### PER.C6 Used for Influenza A Virus Propagation.

It was verified whether replication and propagation of influenza virus could be supported by PER.C6. On the day of infection, PER.C6 cells were seeded in 490 cm<sup>2</sup> tissue culture roller bottles, with the density of 2x10<sup>5</sup> cells/ml in a final volume of 40 ml, in the presence of 5 µg/ml of trypsin-EDTA (Gibco-BRL). Cells were either mock inoculated or infected with the H3N2 strain A/Shenzhen/227/95 (titer 1.5x10<sup>6</sup> pfu/ml) (obtained from Dr. E. Claas, Leiden University Medical Centre, The Netherlands). Infections were performed at moi 10<sup>-4</sup> and 10<sup>-3</sup> pfu/cell. After 1 h of incubation at 37°C, the inoculum was removed by spinning down the cells at 1500 rpm and resuspending the cells in fresh culture medium + 5 µg/ml of trypsin-EDTA. Harvest of 1.3 ml of cell suspension was carried out each day, from day 1 to day 6 post-infection. Supernatants were stored at -80°C and used for hemagglutination assays. Cell pellets were used for direct immunofluorescence tests and for propidium iodide staining.

### Example 4

#### Permissiveness of PER.C6 for Different Influenza Strains

To further investigate the permissiveness of PER.C6 for propagation of various influenza strains, an infection by using the H1N1 vaccine strains A/Beijing/262/95 and its reassortant X-127, obtained from the National Institute for Biological Standards and Control (NIBSC, UK) was performed. On the day of infection, PER.C6 cells were seeded in 490 cm<sup>2</sup> tissue culture roller bottles, with the density of approximately 1x10<sup>6</sup> cells/ml in a final volume of 50ml. Cells were inoculated with 5µl (10<sup>-4</sup> dilution) and 50 µl (10<sup>-3</sup> dilution) of virus in the presence of 5 mg/ml trypsin-EDTA. In order to establish if trypsin was indeed required, one more infection was carried out by inoculating 5µl of the strain A/Beijing/262/95 in the absence of the protease. After approximately 1 h of incubation at 37°C, the inoculum was removed by spinning down the cells at 1500 rpm and resuspending them in fresh culture medium ± 5 mg/ml of trypsin-EDTA. At day 2 and day 4 post-infection more trypsin was added to the

samples. Harvest of 1.3 ml of cell suspension was carried out from day 1 to day 6 post-infection. Supernatants were stored at  $-80^{\circ}\text{C}$  and used for hemagglutination assays and further infections; cell pellets were used for direct immunofluorescence tests. Results obtained with the above mentioned immunofluorescence and hemagglutination assays are shown in FIGs. 4 & 5, respectively, illustrating the efficient replication and release of the viruses.

### Example 5

#### Infectivity of Virus Propagated on PER.C6

It was verified whether the viruses grown in PER.C6 were infectious and if adaptation to the cell line could increase virus yields. Virus supernatants derived from PER.C6 infected with the strains A/Beijing/262/95 and its reassortant X-127 (dil.10-3) and harvested at day 6 post-infection, were used. At the day of infection, PER.C6 were seeded in 490  $\text{cm}^2$  tissue culture roller bottles, with the density of approximately  $1 \times 10^6$  cells/ml in a final volume of 50 ml. Cells were inoculated with 100  $\mu\text{l}$  and 1 ml of virus supernatant in the presence of 5 mg/ml trypsin-EDTA. In order to establish if trypsin was still required, one more infection was carried out by inoculating 100  $\mu\text{l}$  of the strain A/Beijing/262/95 in the absence of the protease. After approximately 1 hour of incubation at  $37^{\circ}\text{C}$ , the inoculum was removed by spinning down the cells at 1500 rpm and resuspending them in fresh culture medium  $\pm$  5 mg/ml of trypsin-EDTA. At day 2 and day 4 post-infection more trypsin was added to the samples. Harvest of 1.3 ml of cell suspension was carried out from day 1 to day 6 post-infection. Supernatants were stored at  $-80^{\circ}\text{C}$  and used for hemagglutination assays and further infections; cell pellets were used for direct immunofluorescence tests. Results obtained with the above mentioned immunofluorescence and hemagglutination assays are shown in FIGs. 6 and 7. Data obtained with the present experiment showed infectivity of the viruses grown in PER.C6 as well as an increase in virus yields.

## Example 6

### The Presence of Cell Surface Receptors for Influenza Virus on PER.C6.

Propagation of human Influenza A and B strains in embryonated chicken eggs leads to a selection of receptor-binding variants that harbor amino acid substitutions at the distal portion of the HA globular head in the exposed and functionally important regions of the molecule. Because of these mutations, the egg-adapted strains can differ from the original human viruses in their antigenic and immunogenic activities, as well as their virulence. Human influenza viruses isolated from MDCK cells usually present a HA protein that is identical to the HA protein present on the virus of the original clinical sample. A recent study (Govorkova 1999) clarified the molecular basis for the selection of variants in chicken eggs and the absence of this variant selection phenomenon in MDCK cells. All human Influenza A and B strains isolated from MDCK cells were found to bind with high affinity and specificity for alpha2,6 sialic acid-galactose linkages present in oligosaccharides present in cell surface receptors, whereas their egg-grown counterparts showed an increased affinity for the alpha2,3 sialic acid-galactose linkages in cell surface receptors carrying oligosaccharides (Sia2-3Gal). Using specific lectins, it was demonstrated that only Sia2-3Gal-containing receptors were present on the surface of chicken embryonic cells, whereas MDCK cells expressed both Sia2-6Gal and Sia2-3Gal. The expression of the Sia2-3Gal and Sia2-6Gal moieties on the surface of PER.C6 cells were studied by FACS analysis, using two different digoxigenin (DIG)-labelled lectins: Sambuca nigra agglutinin (SNA) that specifically recognises Sia2-6Gal linkages and the Maackia amurensis agglutinin (MAA), that specifically recognises Sia2-3Gal linkages. FIG. 8A shows the recognition of the SNA and MAA lectins and their binding to the glycosylation sites. Furthermore, FIG. 8A shows the schematic interaction between the FITC labelled anti-DIG antibody and the DIG-labelled lectin that recognises the specific sialyl bond in the glycosylation backbone of the receptor present on the cell surface. Both lectins were taken from the glycan differentiation kit (Boehringer-La Roche).

The experiment was carried out on PER.C6 cells in suspension and adherent MDCK and CHO cells. MDCK and CHO cells were released from the solid support using trypsin-EDTA (Gibco-BRL). The cell suspensions were then washed once with Mem-5% FBS and incubated in this medium for 1 hour at 37°C. After washing with PBS (Gibco-BRL), the cells were resuspended to a concentration of approximately  $10^6$  cells/ml in binding medium (Tris-buffered saline, pH 7.5, 0.5%BSA, and 1 mM each of  $MgCl_2$ ,  $MnCl_2$  and  $CaCl_2$ ). Cell aliquots were incubated for 1 h at room temp. with the DIG-labelled lectins SNA and MAA. After 1 h, lectin-treated cells were washed with PBS and incubated for an additional hour at room temperature with FITC-labeled anti-DIG antibody (Boehringer-Mannheim). Finally, the cells were washed with PBS and analysed by fluorescence activated cell sorting using a FAC-sort apparatus (Becton Dickinson). The results shown in FIG. 8B demonstrate that PER.C6 cells were stained by both lectins showing the presence of the Sia2-6Gal as well as the Sia2-3Gal receptors.

In the same experiment, MDCK cells were used as positive control for both the sialylated receptors, whereas CHO cells, due to the absence of the alpha 2-6 sialyltransferase glycosylation enzyme in these hamster cells, represented a negative control for the Sia2-6Gal moiety. The upper panels show results with the SNA lectin and the lower panels showing results with the MAA lectin. From these results, it can be concluded that PER.C6 expresses cell surface proteins that have both Sia2-3Gal and Sia2-6Gal linkages in their oligosaccharide chains.

### Example 7

#### **Effect of Different Concentrations of Trypsin-EDTA on the Viability of PER.C6 Cells, on the Influenza Virus Production in PER.C6 Cells and on the Ha Protein Derived Thereof.**

Due to the absolute trypsin requirement for the propagation of influenza viruses in cell cultures, the effects of different concentrations of trypsin-EDTA on PER.C6 cell viability and



virus replication in PER.C6 cells after infection using several Influenza strains were investigated.

Infection with Influenza virus strain A/Sydney/5/97 in the presence of low concentrations of trypsin

5 On the day of infection, PER.C6 cells were seeded in 490 cm<sup>2</sup> tissue culture roller bottles, at a density of 1x10<sup>6</sup> cells/ml, in the presence of trypsin-EDTA at final concentrations of 0.5, 1, 2, 3 and 5 mg/ml.

These trypsin concentrations did not interfere with the growth characteristics of the cells and their viability (data not shown). Cells were either, mock infected or infected with  
10 PER.C6-grown Influenza virus A/Sydney/5/97 at an moi of 10<sup>-4</sup> pfu/cell. The viral production was monitored by direct immunofluorescence (data not shown), hemagglutination assays, single-radial-immunodiffusion (SRID) above and plaque assays, all as described above. Results from this experiment are depicted in FIG. 9 and show that the HA content as measured by SRID as well as the biological activity of the virus, expressed in HAU, were highest when a  
15 trypsin concentration of 1 mg/ml was used. FIG. 9 also shows that by using a plaque assay the highest number of plaque forming units (pfu) per ml was observed in the sample corresponding to cells grown in medium containing 2 mg/ml of trypsin.

Infection with Influenza virus strain B/Harbin/7/94.

On the day of infection, PER.C6 cells were seeded in 490 cm<sup>2</sup> tissue culture roller  
20 bottles at a density of 1x10<sup>6</sup> cells/ml, in the presence of different concentrations of trypsin-EDTA, ranging from 1 to 5 mg/ml. Cells were infected with PER.C6-grown virus B/Harbin/7/94 at an moi of 10<sup>-3</sup> pfu/cell. Production of the virus was monitored by direct immunofluorescence, hemagglutination and plaque assays as shown in FIG. 10. The infectability of PER.C6 at day 2 increased with the concentration of trypsin. At day 3 however,  
25 no significant difference was observed in the percentage of infected cells when 1, 2.5 or 5 mg/ml trypsin was present. In the absence of trypsin (0 µg/ml), no influenza virus infection was detected. At the day of the last harvest (day 4 post-infection), the biological activity of the

virus, as measured by hemagglutination assay, did not differ significantly. Interestingly, the infectivity assay performed in samples that were taken at days 3 and 4 after infection, showed a difference in the production of the virus. The highest titers were obtained at day 3 and day 4 when a trypsin concentration of 2.5 to 5 (day 3) and 1 mg/ml (day 4) were used.

5 Infection with Influenza virus reassortant X-127.

On the day of infection, PER.C6 cells were seeded in T25 tissue culture flasks, at a density of  $1 \times 10^6$  cells/ml, in the presence of different concentrations of trypsin-EDTA ranging from 0 to 7.5 mg/ml. Cells were infected with PER.C6-grown virus X-127 (egg-reassortant for the strain A/Beijing/262/95) at an moi of  $10^{-4}$  and  $10^{-3}$  pfu/cell. Viral growth was monitored by  
10 direct immunofluorescence, hemagglutination and plaque assays. As shown in FIG. 11 and FIG. 12, HAU titers were identical between samples, independent of the trypsin concentration and the initial moi that was used. Furthermore, no significant differences were observed in the infectivity titers, as measured by plaque assay.

15 Infection of PER.C6 with Influenza virus strain A/Sydney/5/97 in the presence of high concentrations of trypsin

To test the effect of increasing concentrations of trypsin on viability of the cells and virus replication, PER.C6 cells were seeded in roller bottles at a density of  $1 \times 10^6$  cells/ml in the presence of various concentrations of trypsin-EDTA ranging from 0 to 12.5  $\mu$ g/ml. Cells were either mock infected or infected with PER.C6 grown virus A/Sydney/5/97 virus at an  
20 moi of  $4 \times 10^{-5}$  pfu/cell. HAU's present in the obtained batches were determined as described. Importantly, data depicted in FIG. 13 clearly show that trypsin concentrations up to 10  $\mu$ g/ml do not interfere with the cell viability. Moreover, the biological activity of the virus obtained at day 4 after infection as measured by HAU was higher when a trypsin concentration of 2.5 to 5  $\mu$ g/ml was used. Furthermore, the TCID<sub>50</sub> was measured (FIG. 14, graph portion A) and  
25 plaque assays were performed (data not shown). No relevant differences were found in these plaque assays, in the infectivity titers (TCID<sub>50</sub>), in the HA cleavage and quantity (approximately 10  $\mu$ g/ml) as determined by western blot analysis shown in FIG. 14B.

### Example 8

#### **Influenza virus production on PER.C6 cells in a hollow fiber-perfusion bioreactor system.**

5 The scalability of influenza virus production in suspension growing PER.C6 cells was studied by using 3 liter (total volume) bioreactors containing a 2 liter cell suspension volume in serum free medium, which is also free of animal or human derived proteins (ExCell 525, JRH Biosciences).

Influenza infection was carried out at a cell density of approximately  $3 \times 10^6$  cells/ml. Cells were inoculated with PER.C6-grown A/Sydney/5/97 virus, at an moi of  $10^{-4}$  pfu/cell.  
10 Samples of 5 to 10 ml of cell suspensions were taken every day to perform general cell counts, to determine the viability of the cells, for glucose concentration measurements, for direct immunofluorescence, for hemagglutination and for infectivity assays. The results of these experiments are shown in FIG. 15.

To investigate the presence and the status of the HA protein western blots using two  
15 different anti-HA antibodies obtained from NIBSC were used. SRID assays as described above were also performed. The results depicted in the two western blots in FIG. 16 show that the Influenza virus batch produced in this bioreactor yielded an HA content of an estimated concentration of 15  $\mu\text{g/ml}$  which was confirmed by SRID assays. The HA produced is comparable to reference NIBSC HA in terms of subunit composition and immune reactivity  
20 with the reference subtype specific antisera.

### Example 9

#### **Infection of PER.C6 with A/Sydney/5/97 in a 15 liter bioreactor followed by a specific Down Stream Process (DSP).**

25 Suspension growing PER.C6 cells were incubated at  $37^\circ\text{C}$  in a 15 liter bioreactor hollow fiber perfusion system, with a cell suspension volume of 10 liter in serum-free ExCell 525 medium (JRH Biosciences). Influenza infection was carried out at  $35^\circ\text{C}$  at a cellular

density of approximately  $3.3 \times 10^6$  cells/ml in medium containing 5 mg/ml trypsin-EDTA (Life Technologies). Cells were inoculated with PER.C6-grown A/Sydney/5/97 virus (passage number 3) at an moi of  $10^{-4}$  pfu/cell. Perfusion with serum-free ExCell 525 medium containing trypsin-EDTA was continued during the first 24 h upon infection. Two days post-infection, 5 cells were fed with a fed-batch solution containing glucose, essential amino acids and extra glutamine: 82 ml per liter suspension containing 50m/v% glucose (NPBI-The Netherlands), 50x essential ammino acids without Gln (Gibco-BRL-Life Technologies) and 200 mM glutamine (Gibco-BRL-Life Technologies). Cell suspension samples of 10 ml were taken every day in order to perform standard cell counts (results shown in FIG. 17, left graph), 10 glucose concentration measurements (results shown in FIG. 17, right graph), direct immunofluorescence (FIG. 18), hemagglutination (FIG. 19) and infectivity assays (data not shown). Furthermore, the HA protein was investigated by western blot analysis and compared to a NIBSC standard HA control (FIG. 20). On the day of the final harvest of the entire cell suspension (92 h post infection), a cell debris clarification was performed in a continuous flow 15 at 20,000g using the Powerfuge™ separation system (Carr, JM Separations) according to the protocols provided by the manufacturer. Clarified supernatant was then concentrated twenty fold using a hollow fiber membrane cartridge of 500 kD cut off (A/G Technology, JM Separations). The results depicted in FIG. 21 show that the quantitative recovery of live influenza virus after concentration by hollow fiber as measured by hemagglutination and 20 infectivity assays is very significant.

### Example 10

#### **The Immunogenicity of PER.C6-grown Influenza Viruses and Vaccines Derived Therefrom.**

25 To determine the immunogenicity of PER.C6 grown influenza viruses an *in vivo* study and challenging model in ferrets was designed. Two batches of trivalent whole-inactivated influenza vaccine (composed of A/Sydney/5/97, A/Beijing/262/95 and B/Harbin/7/94),

containing 15 µg HA of each of the three strains, were used. The first batch was obtained from fertile hens' eggs and the second was obtained from PER.C6 cells. Production, purification, inactivation and formulation of the trivalent whole-inactivated PER.C6-derived Influenza vaccines were performed as described below.

#### 5 Growth of A/Sydney/5/97, A/Beijing/262/95 and B/Harbin/7/94 Influenza strains on PER.C6.

Production of all three influenza viral batches were performed in three separate 3 liter hollow fiber fed-batch bioreactor systems with cell suspension volumes of 2 liter. Fed batch was performed with the addition of the following solution: A total volume of 96 ml containing 50m/v% glucose (NPBI), 50x essential amino acids without Gln (Gibco-BRL-Life Technologies), 200 mM glutamine (Gibco-BRL-Life Technologies) and 7.5 m/v% NaHCO<sub>3</sub> (Merck) was added once. Influenza infections were carried out at cell densities ranging from 1.8x10<sup>6</sup> to 2.6x10<sup>6</sup> viable cells/ml, in ExCell 525 serum free medium containing 5 mg/ml trypsin-EDTA. PER.C6 cells were inoculated with the PER.C6-grown A/Sydney/5/97, A/Beijing/262/95 and B/Harbin/7/94 virus batches at different moi's: 10<sup>-4</sup> (A/Sydney/5/97) or 10<sup>-3</sup> (A/Beijing/262/95 and B/Harbin/7/94) pfu/cell. During the virus production period, samples of 10 ml were taken every day to perform standard cell and viability counts, glucose concentration measurements, direct immunofluorescence and Hemagglutination assays. FIG. 22 (results from the A/Sydney/5/97-infected PER.C6 cells) shows the total and viability cell counts after infection with the virus (upper left panel), the glucose consumption (upper right panel), the percentage of positive cells in the direct immunofluorescence detection (lower left panel) and the HAU's (lower right panel). FIG. 23 (results from the A/Beijing/262/95-infected PER.C6 cells) shows the total and viability cell counts after infection with the virus (upper left panel), the glucose consumption (upper right panel), the percentage of positive cells in the direct immunofluorescence detection (lower left panel) and the HAU's (lower right panel). FIG. 24 (results from the B/Harbin/7/94-infected PER.C6 cells) shows the total and viability cell counts after infection with the virus (upper left panel), the glucose consumption (upper right panel), the percentage of positive cells in the direct immunofluorescence detection (lower

left panel) and the HAU's (lower right panel). Virus containing concentrates were stored at -80°C until DSP.

In all three cases, the glucose consumption and viability and total cell counts of the PER.C6 cells were comparable. Also the production levels of the three viruses, as measured by direct immunofluorescence were similar. Although the HAU and infectivity titers differed between different strains, PER.C6 sustained replication of all influenza viruses that were tested here.

On the day of harvest of the entire batch (either at day 3 or at day 4 post-infection) viral supernatants were clarified by centrifugation at 2000 rpm in a table top centrifuge and concentrated ten fold by ultra filtration using a hollow fiber membrane cartridge of 750 kD cut-off (A/G Technology, JM Separations) following the protocols provided by the manufacturer. Influenza viruses were purified from the concentrated supernatants via two subsequent density centrifugation steps: a 25-70% block sucrose gradient (1.5hrs at 27K) followed by a continuous 25-70% sucrose gradient (4hrs at 23K). Viral bands were diluted in approximately 50 ml of a Phosphate buffer and finally pelleted at 24,000 rpm in an ultracentrifuge. Viral pellets were dissolved in 1.5 to 2.3 ml of a Phosphate buffer, aliquoted and frozen at -80°C.

The formulation of inactivated Influenza vaccines is based on the amount (in micrograms) of the "immunologically active" HA protein, as measured by the SRID assay (Wood et al. 1977). The test was performed to characterize the HA content of the batches. At the same time total amount of proteins was measured using the Lowry-based DC-protein assay kit (Biorad) following the procedures suggested by the manufacturer. It was found that HA constitutes about 20 to 30% of the total protein content of the virus preparation.

## Example 11

### ***In vivo* Immunogenicity of Inactivated Vaccines Produced in Eggs and on PER.C6.**

Ferrets and mice represent two well-established animal models for studying influenza infection, and have been used to determine the efficacy and immunogenicity of influenza vaccines. Using the mouse model test system, the immunogenicity produced by the PER.C6 and egg-derived trivalent vaccines containing A/Sydney/5/97, A/Beijing/262/95 and B/Harbin/7/94 are compared by analyzing sera of vaccinated animals by Hemagglutination inhibition assay. Using the ferret infection model, immunization is followed by a challenge with A/Sydney/5/97. Virus recovery on MDCK cells and Hemagglutination inhibition assay performed on the sera are used to compare the immunogenicity and efficacy of the two vaccines.

#### ***In vivo* study in mice.**

Ninety female Balb/C mice are divided into nine groups of ten mice. On day 0, up to 100 ml of blood is collected. The serum is separated and stored at  $-20^{\circ}\text{C}$ . Each mouse is then vaccinated with the appropriate vaccine according to the schedule in Table I. On day 28, a further 100 ml of blood is taken. Serum is stored at  $-20^{\circ}\text{C}$ . Each mouse is again vaccinated according to the schedule in Table I. On day 42, a 100 ml blood sample is taken and all mice are sacrificed. Serum is separated and frozen at  $-20^{\circ}\text{C}$ . Hemagglutination Inhibition (HI) assays are conducted on serum samples from day 0, 28 and 42. All these assays are conducted in parallel for each day for both egg and cell grown viruses.

Table I

Immunogenicity test in mice.

GROUP NUMBER	ANTIGEN TYPE	IMMUNIZATIO N VOLUME (ml)	VACCINATION ROUTE	TOTAL mg HA per dose
1	Egg trivalent whole virion	0.5	s.c.	9.0
2	Egg trivalent whole virion	0.5	s.c.	3.0
3	Egg trivalent whole virion	0.5	s.c.	1.5
4	Egg trivalent whole virion	0.5	s.c.	0.15
5	PER.C6 trivalent whole virion	0.5	s.c.	9.0
6	PER.C6 trivalent whole virion	0.5	s.c.	3.0
7	PER.C6 trivalent whole virion	0.5	s.c.	1.5
8	PER.C6 trivalent whole virion	0.5	s.c.	0.15
9	PBS	0.5	s.c.	0

*In vivo* study in ferrets.

Eighteen adult female ferrets (albino or polecat) were divided in three groups of six divided as follows: Group 1 received the egg-derived test vaccine intramuscularly (IM), the animals were challenged with A/Sydney/5/97. Group 2 received the PER.C6 derived test vaccine IM, the animals were challenged with A/Sydney/5/97. Group 3 received the test vaccine diluent only and were challenged with A/Sydney/5/97. On days 0 and 28, the test vaccines were administered. On day 56, all the ferrets were infected intranasally with 0.5 ml of the A/Sydney/5/97 challenge virus at TCID<sub>50</sub> 10<sup>3</sup>. Nasal washes were performed and inflammatory cell counts, temperature and weights of the ferrets were monitored once daily from day 57 to 63. All animals were sacrificed on day 63. Serum was separated and stored at -20°C. The nasal wash samples were stored on ice and a nasal wash recovery cell count was performed using Trypan blue exclusion assay.

The titer of the virus obtained from the nasal wash samples was determined by measuring the virus recovery on MDCK cells. The Spearman and Karber (1931) calculation was used to calculate TCID<sub>50</sub> values. Hemagglutination inhibition analyses were conducted on



serum samples taken on day 0, 28, 56 and 63. From this experiment, it was concluded that the PER.C6 derived test vaccine was effective.

### Example 12

#### 5 **Characterization of HA protein derived from Influenza virus produced on PER.C6.**

In order to study the glycosylation of HA in PER.C6 cells, a batch of uncleaved HA (HA0) was generated. PER.C6 cells were infected with virus A/Sydney/5/97 (passage number 5 on PER.C6) at moi's of 1, 0.1 and 0.01 pfu/cell in ExCell 525 medium containing trypsin-EDTA at the final concentration of 5 mg/ml. After 1 h of incubation at 35°C, cells were  
10 washed twice with PBS to remove trypsin and incubated O/N at 35°C and 10% CO<sub>2</sub>, in the absence of trypsin. The next day, cell suspensions were harvested and centrifuged (500g) and cell pellets were washed twice with medium. Viral supernatants were frozen at -80°C and samples thereof were used in western blot assays as described to investigate the presence or absence of uncleaved HA protein. Uncleaved HA protein (HA0) consists of the two subunits:  
15 HA1 and HA2, that are connected via a disulfide bond. Since this disulfide bond can be disrupted by reduction with DTT, HA1 and HA2 can be separated and visualized on a reducing gel followed by western blots using antisera that recognize the subunits. If the HA protein consists only of HA0, one band will be visible that migrates slower through an SDS/PAGE gel as compared to the HA1 subunit and the fastest migrating HA2 subunit. The  
20 results shown in FIG. 25 suggest the presence of mainly uncleaved HA0 from PER.C6 infections when compared to the egg-derived positive control that was obtained from the NIBSC (UK). To confirm that the band detected was indeed uncleaved hemagglutinin, digested an HA0 sample was digested with different concentrations of trypsin ranging from 2.5 to 10 µg/ml in medium O/N at 37°C. The digested proteins were then loaded under  
25 reducing conditions on an SDS/PAGE gel followed by western blot analysis using the same antisera as described for FIG. 14. As shown in FIG. 26A, cleavage of the HA0 could be achieved, confirming the generation of uncleaved HA protein on PER.C6. Based on these

results, an assay to determine trypsin activity in culture medium, using Influenza HA0 as substrate is developed.

#### Trypsin activity assay

To determine whether trypsin, present in the culture medium of an Influenza  
5 production run is still active, a trypsin activity assay has been developed. This assay is based on the measurement of the enzymatic activity of trypsin to cleave the substrate that is most relevant for influenza vaccine production: the HA0.

It was determined whether, in a culture of PER.C6 inoculated with Influenza  
B/Harbin/7/94 (moi  $10^{-3}/10^{-4}$  pfu/cell), the trypsin remained active over the entire production  
10 run. To this end, 10  $\mu$ l of supernatant taken at day 1, 2 and 3 post infection were used to cleave 68 ng of the substrate that consisted of HA0 of Influenza A Sydney/5/97 virus, O/N at 37°C. Following digestion, protease inhibitors were added to a final concentration of 1x (Complete protease inhibitor cocktail, Boehringer Mannheim) in 3x Laemli buffer with 150 mM DTT (Fluka). The samples were loaded on a 10% Tris-HCL SDS/PAGE gel (Biorad) and run. The  
15 western blot was performed as described. The results are shown in FIG. 26B, and demonstrate that in cultures of PER.C6 inoculated with Influenza B/Harbin virus trypsin remained active during the entire production run, as culture supernatants were able to cleave HA0 completely.

### **Example 13**

#### **20 Digestion of HA0 with N-Glycosidase F**

The influenza virus HA protein is a glycoprotein that contains 3 to 9 N-linked  
glycosylation oligosaccharide sites. The number of sites depends on the virus strain. The  
location of these sites is determined by the nucleotide sequence of the HA gene, and since the  
viral genome of Influenza is replicated by an error-prone RNA polymerase, mutations that  
25 generate the addition or removal of glycosylation sites occur at high frequency. The composition and structure of the oligosaccharide chains present on the HA is then determined by the array of biosynthetic and trimming glycosylation enzymes provided by the host cell.

Since glycosylation of HA plays an important role in virulence and vaccine efficacy, the properties of HA produced on Influenza infected PER.C6 was investigated. A digestion of A/Sydney/5/97 uncleaved HA0 protein with the N-glycosydase F enzyme was performed using protocols provided by the manufacturer to remove the seven oligosaccharides expected to be present on the A/Sydney/5/97 HA polypeptide. Influenza A/Sydney/5/97 was lysed with 1% Triton X-100 (Merck). Protease inhibitor was added to an aliquot of this lysed virus corresponding to 68 ng of HA, to a final concentration of 1x (Complete Protease Inhibitor Cocktail Boehringer Mannheim). This sample was incubated in the presence of 100 mM NaPO<sub>4</sub> pH 7, 10mM EDTA (J.T. Baker), 1% SDS (J.T. Baker) and 1% B-mercaptoethanol (Merck). This was incubated for 10 min. at room temperature. The sample was diluted 5 times in mM NaPO<sub>4</sub> pH 7, 10mM EDTA (J.T. Baker), 0.625% NP-40 and 1% B-mercaptoethanol (Merck). Of this, 40 µl was used for the glyco-F digestion. For this, 2 µl 1U/µl of glyco-F (N-Glycosidase F, Boehringer) was added and incubated for a minimum period of 16 h at 37°C. Then 3x Laemli buffer with 150 mM DTT (Fluka) was added to a final concentration of 1x. The samples were run on a 7.5% SDS/PAGE gel. The SDS-Page and western blot were performed as follows. In brief, the blot was blocked for 30 min at room temperature with block solution (5% non fat dry milk powder, Biorad in TBST supplemented with 1% rabbit serum (Rockland), followed by 3 washes with TBST. Then, the blot was incubated with the anti-A/Sydney/5/97 HA antiserum (98/768 NIBSC) diluted 1/500 in 1%BSA/TBST with 5% rabbit serum (Rockland) overnight at room temperature. Again, the blot was washed 8 times with TBST. Finally, the blot was incubated with the rabbit anti sheep antiserum-HRP labelled (Rockland) 1/6000 diluted in block solution for 1 h at room temperature. After 8 washes with TBST the protein-conjugate complex was visualized with ECL (Amersham Pharmacia Biotech), and films (Hyperfilm, Amersham Life Science) were exposed. As shown in FIG. 27, treatment with the glycosidase-F enzyme clearly reduced the size of the protein with approximately 28-30kD, being approximately the predicted loss of about 4 kD per oligosaccharide. The protein band depicted with an asterisks (\*) is the de-glycosylated HA0,

that migrates similarly to the HA1 subunit product obtained after cleavage of HA0 into HA1 and HA2 subunits (right lanes).

#### **Example 14**

##### **5 Cleavage of HA0 with Accutase.**

The possibility of replacing the mammalian-derived trypsin-EDTA with non-mammalian or recombinant proteins was investigated. Recently, a mixture of proteolytic and collagenolytic enzymes (Accutase<sup>TM</sup>, PAA) from invertebrate species became available for routine cell culture. Due to its non-mammalian source, Accutase is free of prions, parvovirus, and other components that potentially can contaminate trypsin-EDTA solutions. No information regarding the type of proteases present in Accutase could be obtained to date. The cleavage of HA0 was studied using western blot analysis. A constant amount of HA0 protein, obtained by PER.C6 infected with A/Sydney/5/97 at an moi 1 pfu per cell without trypsin, was digested with serial dilutions of Accutase, O/N at 37°C. As a positive control the same amount of HA0 digested with 100 ng of trypsin-EDTA was used. The digested proteins were then loaded on a 10% SDS-PAGE gel, under reducing conditions, for western blot analysis. As shown in FIG. 28 digestion with 2 ml of Accutase treatment resulted in complete cleavage of HA0; partial cleavage was observed using 0.2ml. These results suggest that treatment with Accutase during influenza replication and production can replace trypsin-EDTA during influenza infections on PER.C6.

#### **Example 15**

##### **Electron Microscopy Analysis of Influenza Viruses on PER.C6 cells.**

Transmission electron microscopy studies were done on PER.C6 cells that were infected with the influenza strain A/Sydney/5/97 as well as on viral containing supernatants and sucrose purified material to determine the phenotype of this influenza virus produced on PER.C6. All methods that were used are well known to persons skilled in the art. FIG. 29

shows that the last stages of the virus life cycle are represented by budding and release of enveloped virions from the cytoplasmic membrane. Spikes corresponding to the HA and NA viral proteins were detected, ornamenting the periphery of the virion particles. The figure also shows the characteristic pleiomorphism of influenza viruses.

5

### Example 16

#### Infection of PER.C6 with a Large Variety of Influenza A and B Virus Strains

The use of PER.C6 as a platform technology for the production of influenza vaccine would preferably require PER.C6 to support the growth of a wide range of strains of different influenza subtypes.

10

Static suspension cultures of PER.C6 cells that were grown in T25 flasks and/or in 6 well plates in ExCell 525 medium, were infected at a cell density of  $10^6$  cells/ml with 16 different strains of influenza viruses (FIG. 30A). These strains comprised several H3N2, H1N1, B type and Avian strains. Infections were performed in the presence of 5  $\mu$ g/ml of trypsin. The viruses were obtained from NIBSC as egg-passaged wt or reassortant strains and are noted. Infection was performed with a virus dilution recommended by the NIBSC in the product sheets that were delivered with the different strains. All viruses tested were capable of propagation on PER.C6 as visualized by immunofluorescence (data not shown) and titration of supernatant fluids in pfu assay (FIG. 30B).

15

20

These results show that even influenza strains (depicted by an asterisks), such as A/Johannesburg/33/94, B/Beijing/184/ 93 and A/Duck/Singapore-Q/F119-3/97, which are normally very difficult to produce on embryonated eggs, can replicate and be produced on PER.C6 cells.

### Example 17

#### Generation of Herpes Simplex type 1 (HSV-1) virus, Herpes Simplex type 2 (HSV-2) virus and Measles virus on PER.C6

It was tested whether viruses other than influenza virus and adenovirus, for example, Herpes Simplex Virus type 1 and 2 and measles virus, could also replicate on PER.C6. Vaccines that are derived from these PER.C6-grown viruses and that induce neutralizing effects in humans for protection against wt infections are generated from the PER.C6-grown virus batches. The strains that were obtained from ATCC and used for infection of PER.C6 cells are depicted in Table II.

10

Table II

Herpes simplex virus and Measles strains that were obtained from the ATCC and that were used for infection of PER.C6 cells.

15

20

Virus	Strain	ATCC cat no.	Lot no.	Passage history	Titer
Herpes Simplex Type 1	Macintyre	VR-539	1327850	y.s./12, PR RabK/5, Mb/1, PrRabK/5, Vero/4, Vero(ATCC CCl-81)/1	$10^{6.75}$ TCID <sub>50</sub> /200µl
Herpes Simplex Type 2	MS	VR-540	216463	Sheep choroid plexus/?, HeLa/?, PrRabK/7, Vero(ATCC CCl-81)/3	$10^{7.5}$ TCID <sub>50</sub> /200µl
Measles	Edmonston	VR-24	215236	HK/24, HuAm/40, MRC-5/1, MRC-5(ATCC CCL-171)/1	$10^4$ TCID <sub>50</sub> /ml

To test whether HSV-1 and HSV-2 and measles viruses obtained from the ATCC could replicate and be produced on PER.C6, passage number 46 cells were seeded in Labtek chambers, coated with Poly-L-Lysine using known methods, at  $10^5$  cells/well. Monkey-derived Vero cells (obtained from ATCC) were cultured at passage number 137 and were used as positive controls and seeded at a density of  $2.5 \times 10^4$  cells/well. At day 0, when wells with

PER.C6 cells were 60% and Vero cells 80% confluent, cells were infected with different moi's (  $10^{-3}$ ,  $10^{-2}$ ,  $10^{-1}$  and 1 TCID<sub>50</sub> per cell). At daily intervals upon infection, cells were fixed and assayed in immunofluorescence using FITC-conjugated type specific monoclonal antibodies using a kit (Imagen Herpes Simplex Virus (HSV) Type 1 and 2, (Dako) and FITC-conjugated antibodies against the HA and matrix protein of measles virus (measles IFA kit, Light 5 diagnostics), following the procedures suggested by the manufacturer. The antisera are directed against HSV-1 and -2 and Measles virus antigens.

The results summarized in FIG. 31 show that PER.C6 is permissive for HSV-1 (FIG. 31, portion D), HSV-2 (FIG. 31, portion E) and measles virus (FIG. 31, portion A) infections. 10 Furthermore, the kinetics suggest that these viruses replicate on PER.C6 in an moi-dependent manner.

Next, it was investigated whether HSV-1, -2 and measles virus could be propagated on PER.C6. To this end, cells were infected with moi of 0.01, 0.1 and 1 TCID<sub>50</sub>/cell for HSV-1 (FIG. 32 lower portion) and HSV-2 (FIG. 32 upper portion) and an moi of 0.001 TCID<sub>50</sub>/cell 15 for measles virus (FIG. 32 middle portion) (passage number 1). At the occurrence of almost complete CPE, cells and supernatants were harvested, quickly frozen in liquid N<sub>2</sub>, and thawed. After this, clarified supernatants were passaged blindly using approximately 100 µl, to PER.C6 (this is passage number 2). After reaching almost complete CPE again, a third passage (passage number 3) was performed in a similar manner. The moi's of the passage number 2 20 and 3 were determined in retrospect by TCID<sub>50</sub> assays.

The results of these experiments show that Herpes Simplex Virus type 1 and -2 and Measles viruses can be replicated on PER.C6 and that replication and propagation can even occur when moi's as low as  $10^{-7}$  are used.

### Example 18

#### Screening of Rotavirus for Replication on PER.C6.

To test whether PER.C6 could also support the replication of a rotavirus, PER.C6 cells were infected with a rhesus rotavirus (MMU 18006; ATCC#VR-954; strain S:USA:79:2; lot # 2181153). PER.C6 cells (passage number 41) were cultured at a density of  $1 \times 10^5$  per ml, and monkey-derived Vero cells (obtained from ATCC, passage number 139) were cultured at a density of  $2.5 \times 10^4$  per ml, and subsequently seeded in Labtek chambers, that had been pre-coated with poly-L-Lysine as previously identified. Cells were infected with an moi of 1 TCID<sub>50</sub>/cell of Rhesus rotavirus in the presence and absence of 2 µg/ml of trypsin-EDTA. After 90 min of infection, cells were washed with ExCell 525 medium and further incubated at 37°C at 10% CO<sub>2</sub> in a humidified atmosphere. On 5 consecutive days following infection, samples of supernatants were harvested, clarified from cells and cell debris by centrifugation at 2000 rpm in a table top centrifuge and analysed in an ELISA specific for rotavirus (IDEIA Rotavirus, Dako). The results depicted in FIG. 33 clearly show that Rhesus rotavirus replicates on PER.C6.

Although the invention has been described with a particular amount of detail and with respect to particular examples, the scope of the invention is to be determined by the appended claims.



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40



## CLAIMS

What is claimed is:

1. A method for producing a desired virus and/or desired viral proteins other than adenovirus or adenoviral proteins for use as a vaccine, said method comprising:

providing a cell with at least a sequence encoding at least one gene product of an adenoviral E1 gene or a functional derivative of said adenoviral E1 gene,

providing said cell with a nucleic acid sequence encoding said desired virus and/or said desired viral proteins,

culturing said cell in a suitable medium and allowing for expression of said desired virus and/or said desired viral proteins, and

harvesting said desired virus and/or desired viral proteins from said suitable medium and/or said cell.

2. The method according to claim 1, wherein said cell is a human primary cell.

3. The method according to claim 1 or 2, wherein said cell is immortalized by a gene product of said adenoviral E1 gene.

4. The method according to any one of claims 1-3, wherein said cell is derived from a human embryonic retinoblast.

5. The method according to any one of claims 1-4, wherein the sequence encoding at least one gene product of the adenoviral E1 gene is present in the genome of said human cell.

6. The method according to any one of claims 1-5, wherein said cell does not produce adenoviral structural proteins.

7. The method according to any one of the preceding claims, wherein said cell further  
5 comprises a sequence encoding adenoviral E2A or a functional derivative or analogue or fragment thereof.

8. The method according to any one of the preceding claims wherein said sequence encoding E2A or a functional derivative or analogue or fragment thereof is present in the  
10 genome of said human cell.

9. The method according to claim 7 or claim 8, wherein said adenoviral E2A encoding sequence encodes a temperature sensitive mutant E2A.

10. The method according to any one of the preceding claims, wherein said cell  
15 comprises no other adenoviral sequences.

11. The method according to any one of the preceding claims, wherein said cell is capable of growing in suspension.  
20

12. The method according to any one of the preceding claims wherein said cell is cultured in the absence of serum.

13. The method according to any one of the preceding claims wherein said cell is  
25 PER.C6 as deposited under ECACC no. 96022940 or a derivative thereof.

14. The method according to any one of claims 1-13, wherein said desired virus and/or said desired viral proteins comprise a protein that undergoes post-translational, peri-translational, or both peri-translational and post-translational modifications.

5 15. The method according to claim 14 wherein said modifications include glycosylation.

16. The method according to any one of the preceding claims wherein said desired viral proteins comprise at least one of an influenza virus neuraminidase and/or a hemagglutinin.

10

17. The method according to any one of claims 1-16, wherein said virus is selected from the group consisting of enterovirus, rhinovirus, aphthovirus, and poliomyelitis virus.

18. The method according to any one of claims 1-16, wherein said virus is selected  
15 from herpesvirus, herpes simplex virus, pseudorabies virus, and bovine herpes virus.

19. The method according to any one of claims 1-16, wherein said virus is selected from the group consisting of orthomyxovirus, influenza virus, paramyxovirus, Newcastle Disease virus, respiratory syncytio virus, mumps virus, and measles virus.

20

20. The method according to any one of claims 1-16, wherein said virus is selected from the group consisting of retrovirus, human immunodeficiency virus, parvovirus, and papovavirus.

25 21. The method according to any one of claims 1-16, wherein said virus is selected from the group consisting of rotavirus, coronavirus, transmissible gastroenteritis virus, flavivirus, tick-borne encephalitis virus, and yellow fever virus.

22. The method according to any one of claims 1-16, wherein said virus is selected from the group consisting of togavirus, rubella virus, and Eastern-, Western-, or Venezuelan Equine Encephalomyelitis virus.

5        23. The method according to any one of claims 1-16, wherein said virus is selected from the group consisting of a hepatitis causing virus, hepatitis A virus, and hepatitis B virus.

24. The method according to any one of claims 1-16, wherein said virus is selected from the group consisting of pestivirus, hog cholera virus, rhabdovirus, and rabies virus.

10

25. A method according to any one of claims 1-16, wherein said virus is a Bunyaviridae virus or Hantavirus.

26. Use of a human cell having a sequence encoding at least one adenoviral E1 protein  
15 or a functional derivative, homologue or fragment thereof in the human cell's genome which human cell does not produce structural adenoviral proteins for the production of a virus or at least one viral protein for use in a vaccine.

27. Use according to claim 26, wherein said human cell is derived from a primary cell.

20

28. Use according to claim 26 or 27, wherein said human cell is a PER.C6 cell or a derivative thereof.

29. Use according to claim 26-28, wherein said cell further comprises a sequence  
25 encoding adenoviral E2A or a functional derivative or analogue or fragment thereof in its genome.

30. Use according to claim 29, wherein said adenoviral E2A is temperature sensitive.

31. A virus or a viral protein for use in a vaccine obtainable by a method according to any one of claims 1-25 or by a use according to any one of claims 26-30, said virus or said viral protein being free of any non-human mammalian proteinaceous material.

32. A human cell having a sequence encoding at least one E1 protein of an adenovirus or a functional derivative, homologue or fragment thereof in its genome, which cell does not produce structural adenoviral proteins and having a nucleic acid encoding a virus or at least one non-adenoviral viral protein.

33. The human cell of claim 32 which is derived from PER.C6 as deposited under ECACC no. 96022940.

34. The human cell of claim 32-33, further comprising, in the human cell's genome, a sequence encoding adenoviral E2A or a functional derivative or analogue or fragment thereof.

35. The human cell of claim 34, wherein said adenoviral E2A is temperature sensitive.

36. A kit for determining activity of a protease in a sample, said kit comprising:  
at least one viral protein or virus obtainable by a method according to any one of claims 1-25 or by a use according to any one of claims 26-30.

37. Use of a viral protein or a virus obtainable by a method according to any one of claims 1-25 or by a use according to any one of claims 26-30 for determining protease activity in a sample.



38. The kit of claim 36 or the use according to claim 37, wherein said protease comprises trypsin.

39. The kit according to claim 36 or The use according to claim 37, wherein said  
5 sample comprises culture medium.

40. A method for concentrating influenza virus under conditions capable of at least in part preserving virus infectivity, said method comprising:

10 obtaining a cell cleared supernatant comprising said virus from a culture of cells, and ultrafiltrating said supernatant under low shear conditions.

41. The method according to claim 40, wherein said low shear conditions are obtained with a hollow fiber.

15 42. The method according to claim 40 or claim 41, wherein said culture of cells comprise *in vitro* cultured cells.

43. The method according to any one of claims 40-42, wherein said ultrafiltration is performed with filter comprising a cut off of 750 KD.

20 44. The method according to any one of claims 40-43, wherein said concentration further comprises at least a partial removal of proteins comprising a molecular weight smaller than 750 KD.

25 45. Infectious influenza virus or derivatives thereof concentrated with a method according to any one of claims 40-44.

## Abstract

Means and methods for producing mammalian viruses, the method comprising infecting a culture of immortalized human cells with a virus, incubating the culture infected with virus to propagate the virus under conditions that permit growth of the virus, and to form a virus-containing medium, and removing the virus-containing medium. The viruses can be harvested and be used for the production of vaccines. Advantages include that human cells of the present invention can be cultured under defined serum free conditions, and the cells show improved capability for propagating virus. Methods are provided for producing, in cultured human cells, influenza virus and vaccines derived thereof. This method eliminates the necessity of using whole chicken embryos for the production of Influenza vaccines. The method also provides for the continuous or batch-wise removal of culture media. As such, the present invention allows the large scale continuous production of viruses to a high titer.

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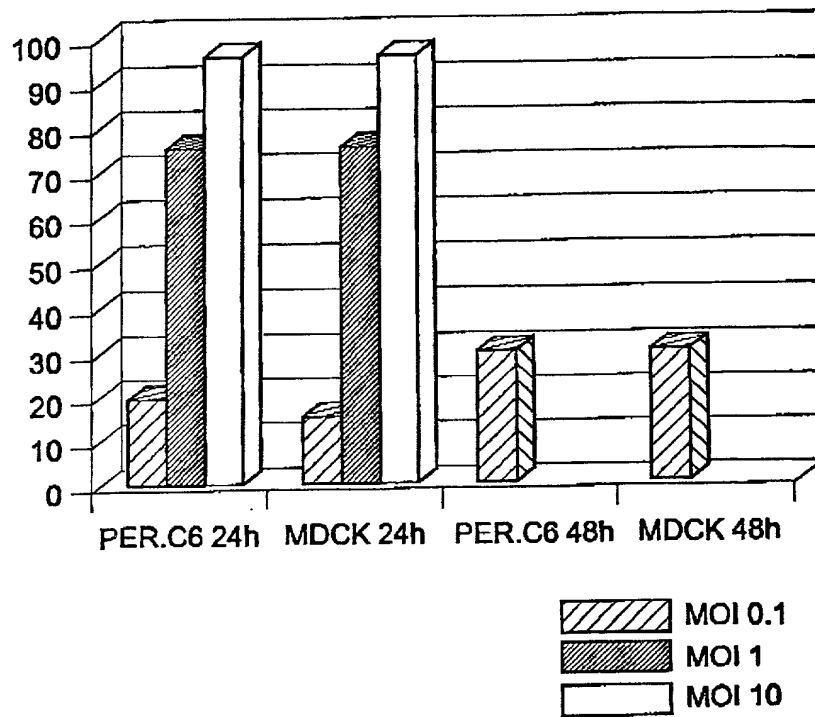


Fig. 1

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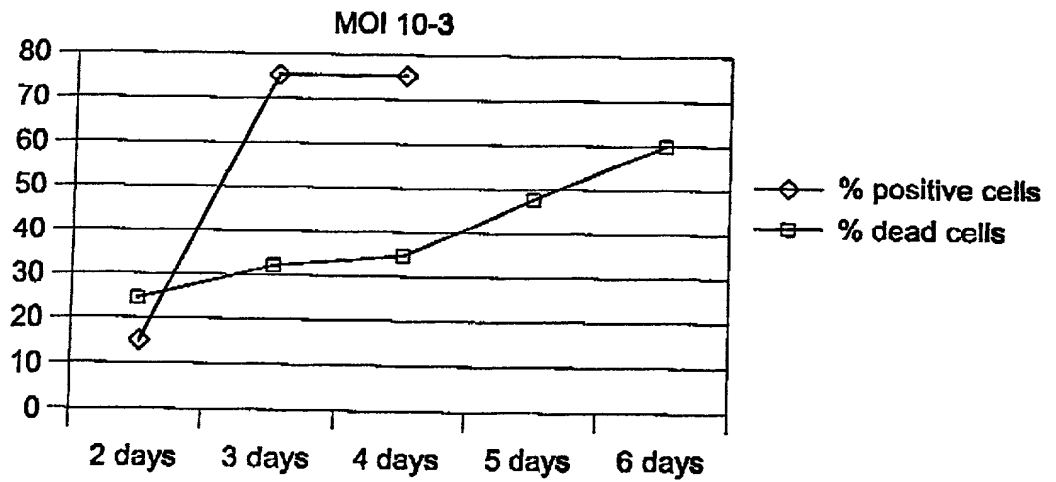
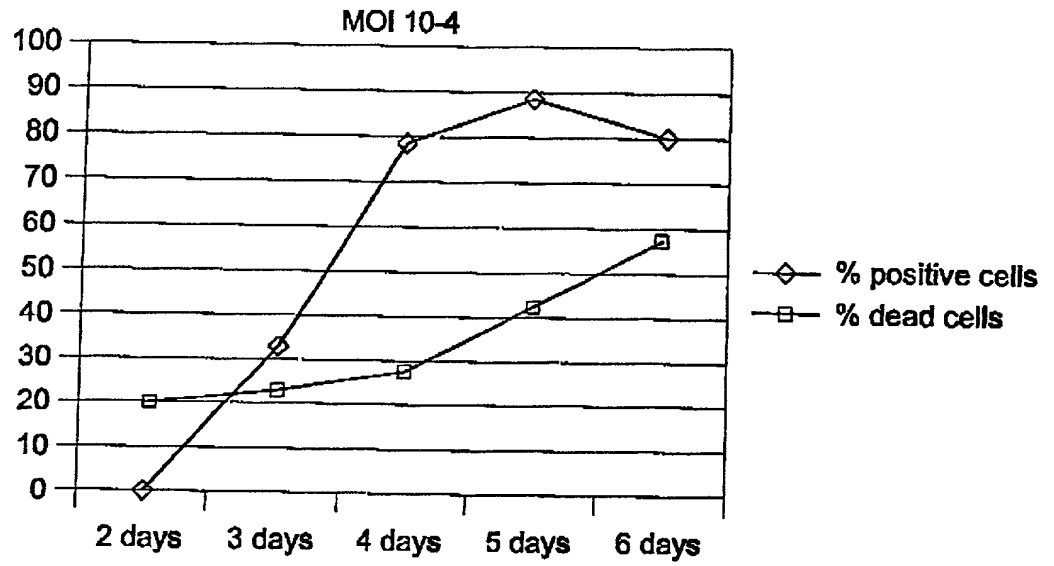


Fig. 2

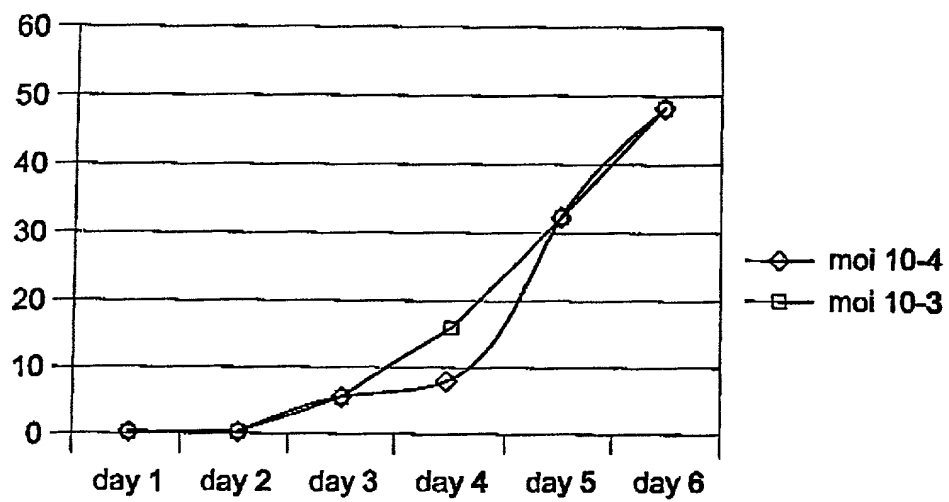


Fig. 3

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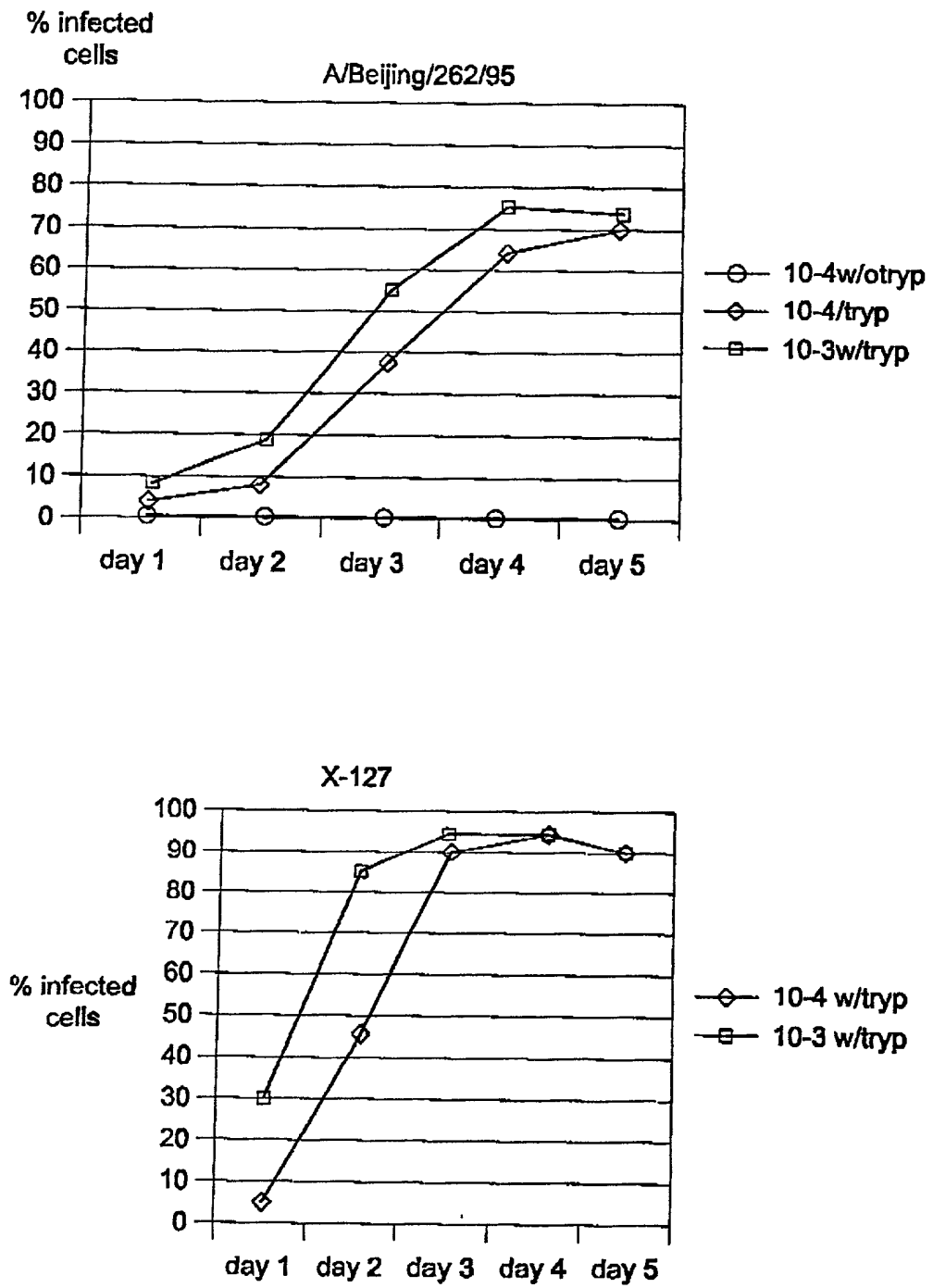


Fig. 4

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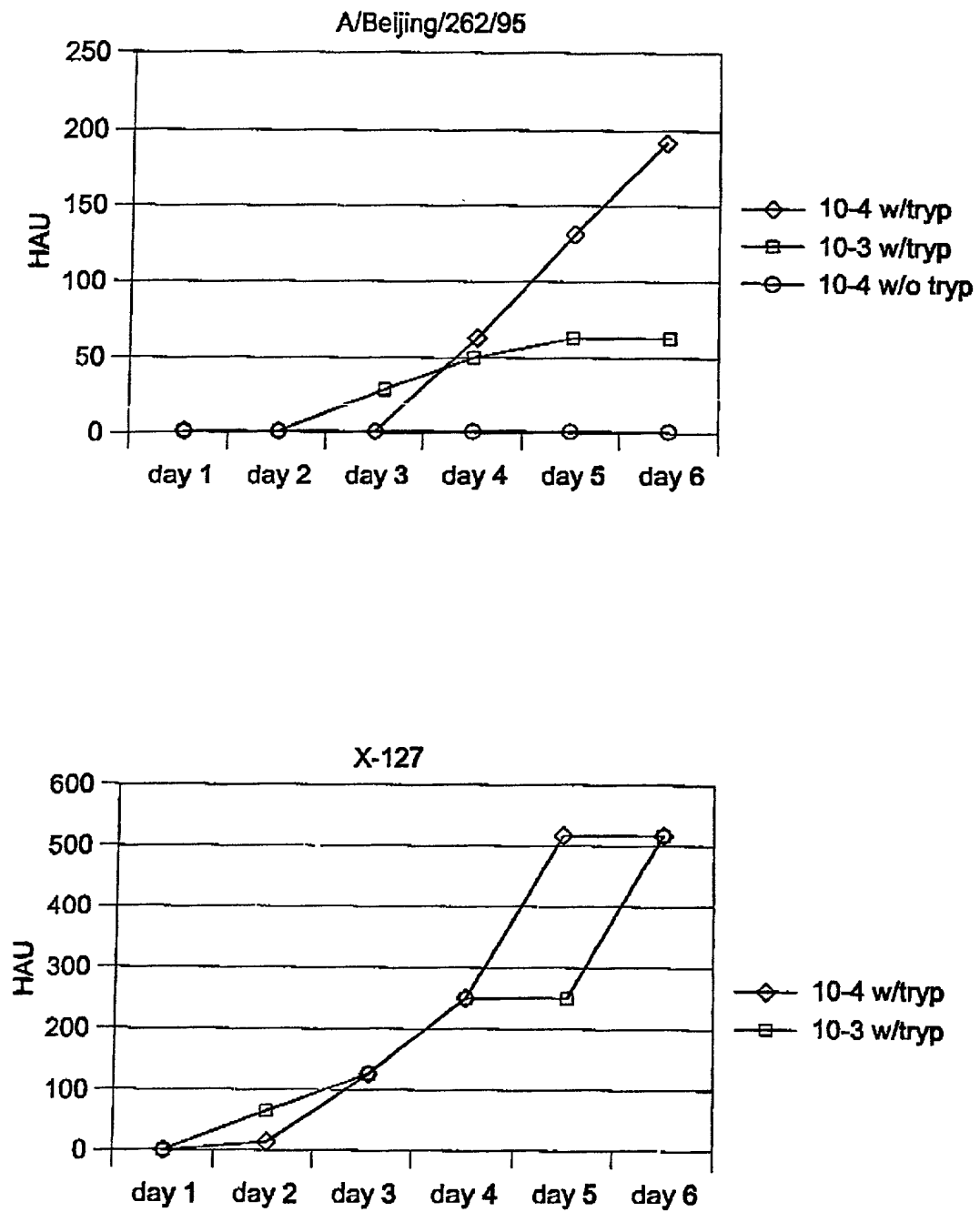


Fig. 5

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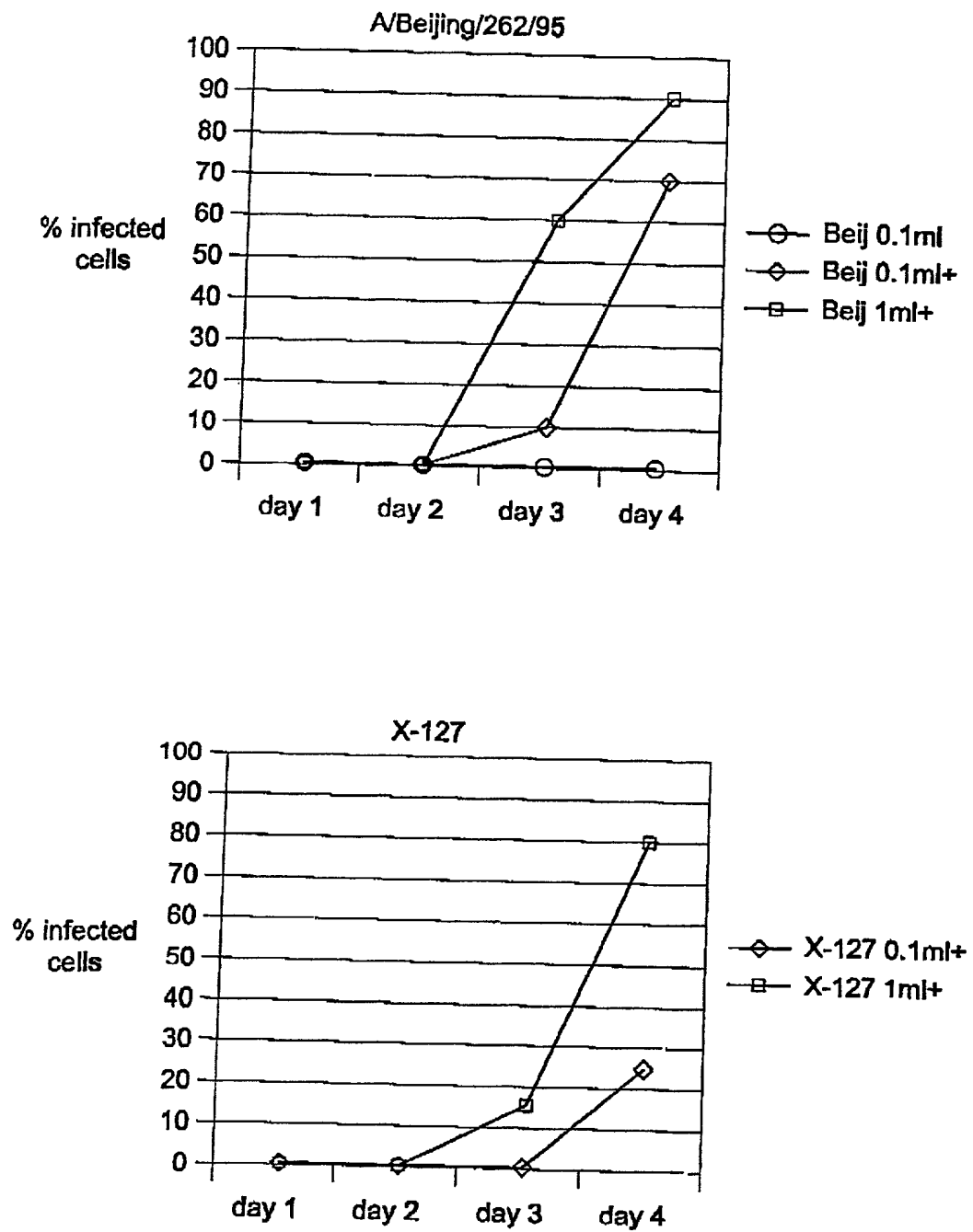


Fig. 6



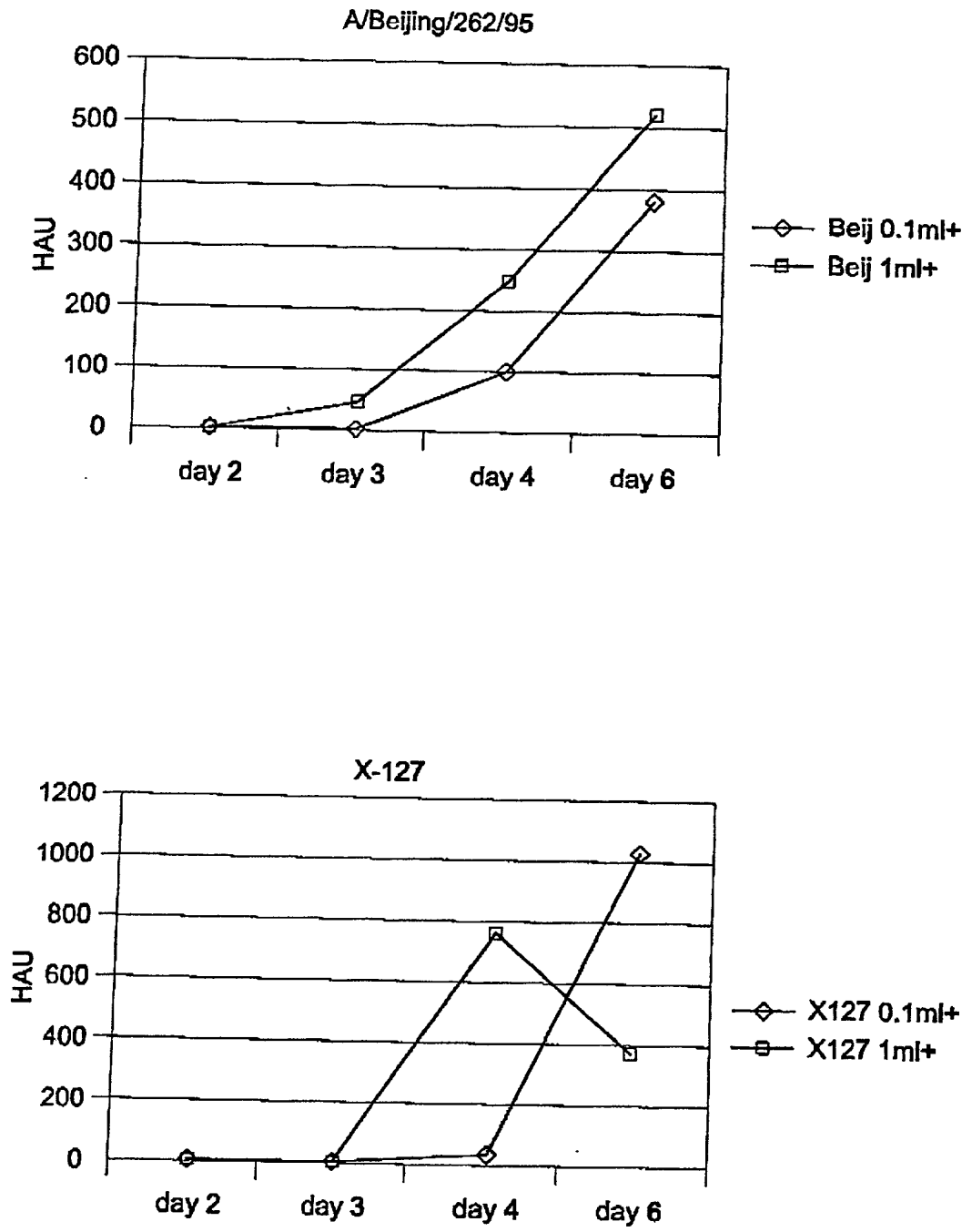
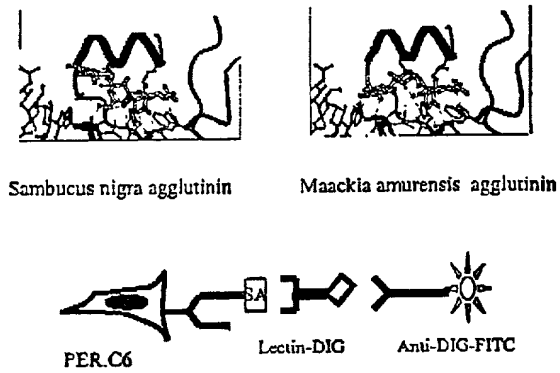


Fig. 7

Fig. 8

A



B

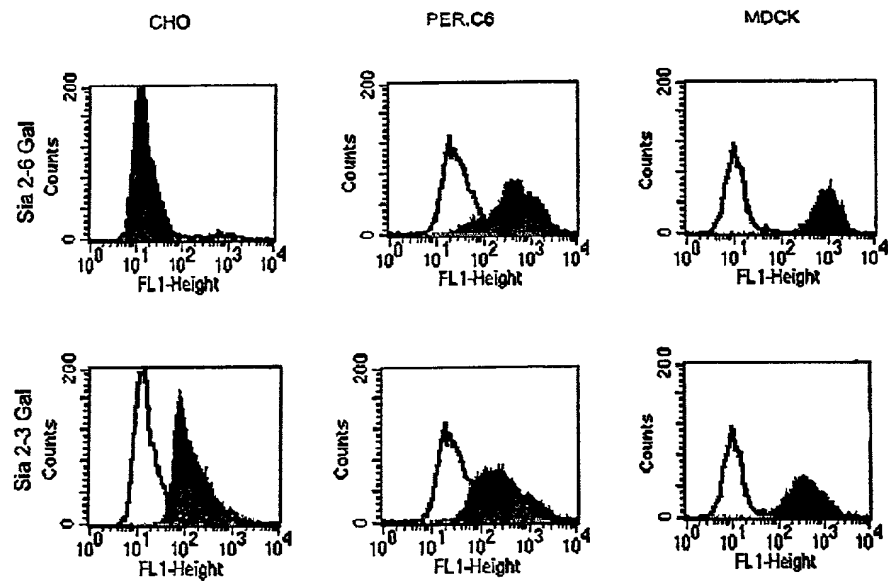


Fig 9

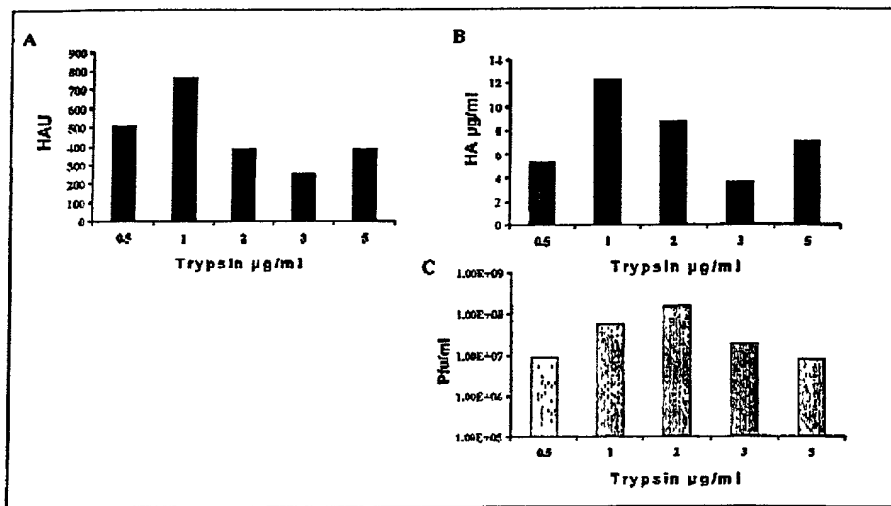


Fig. 10

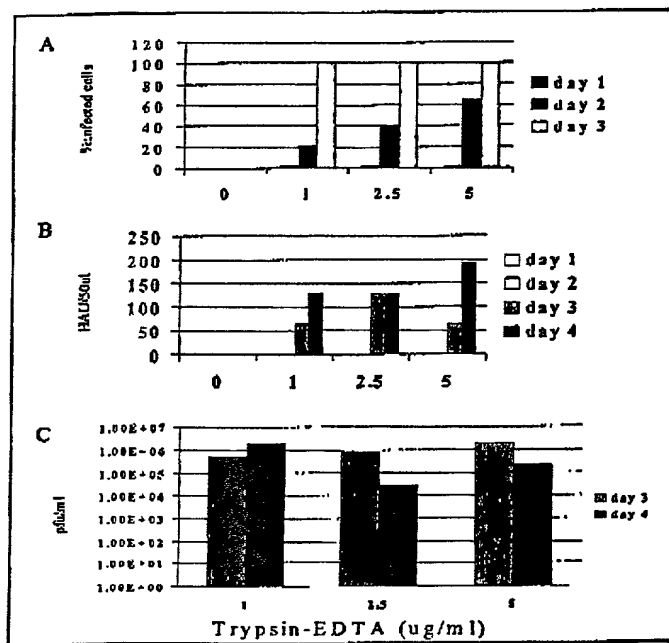


Fig 11

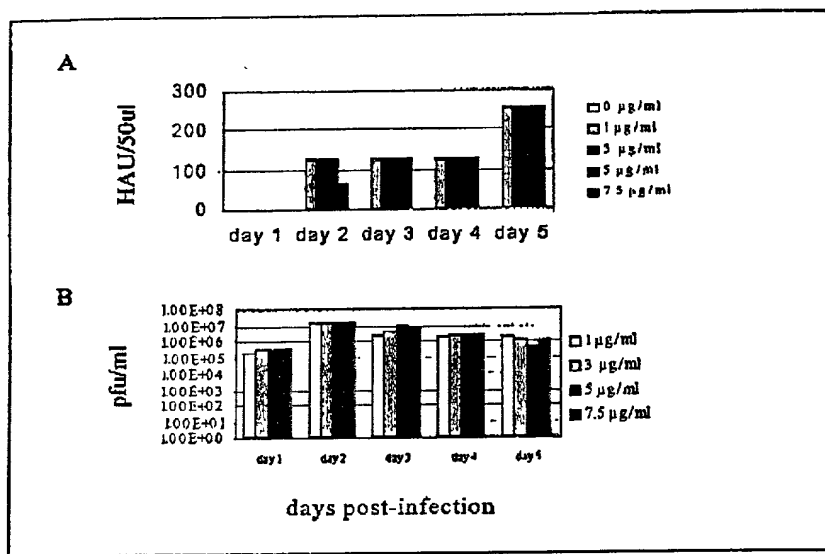


Fig. 12

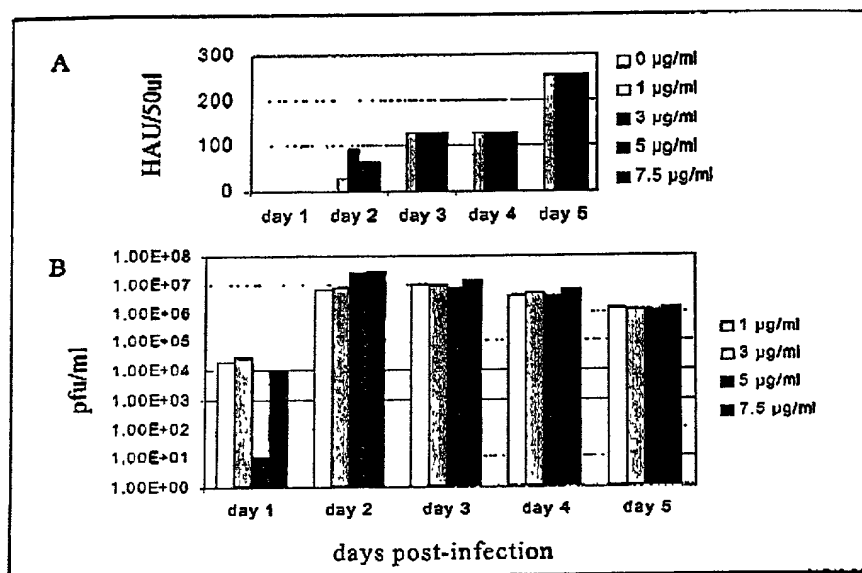


Fig. 13.

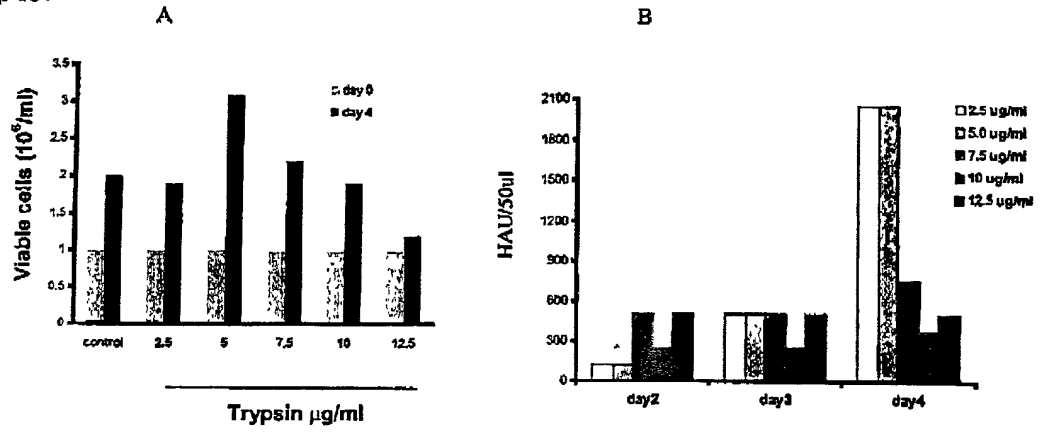


Fig. 14

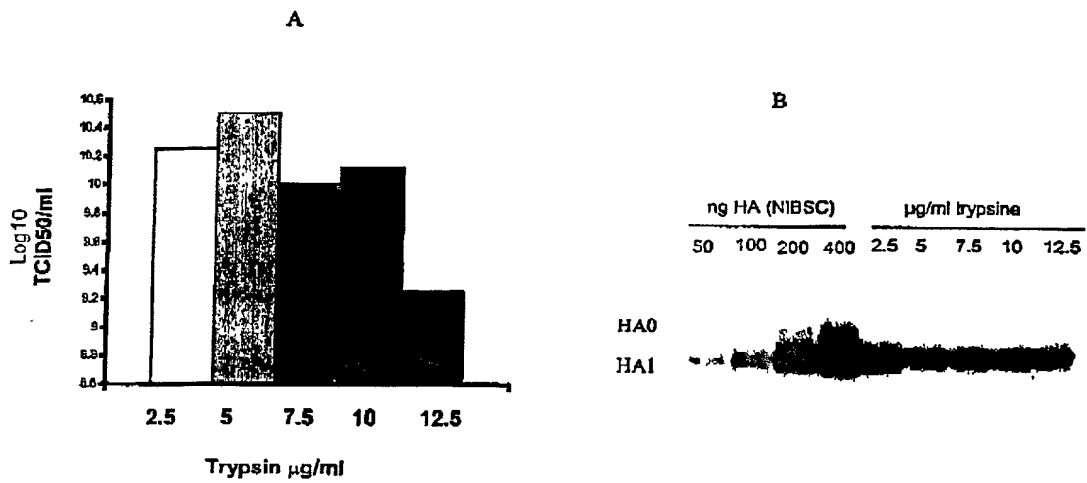


Fig. 15

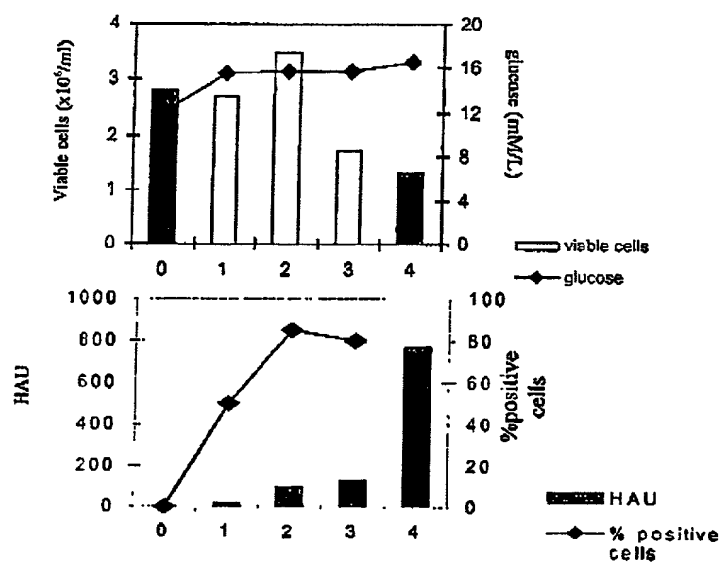


Fig. 16

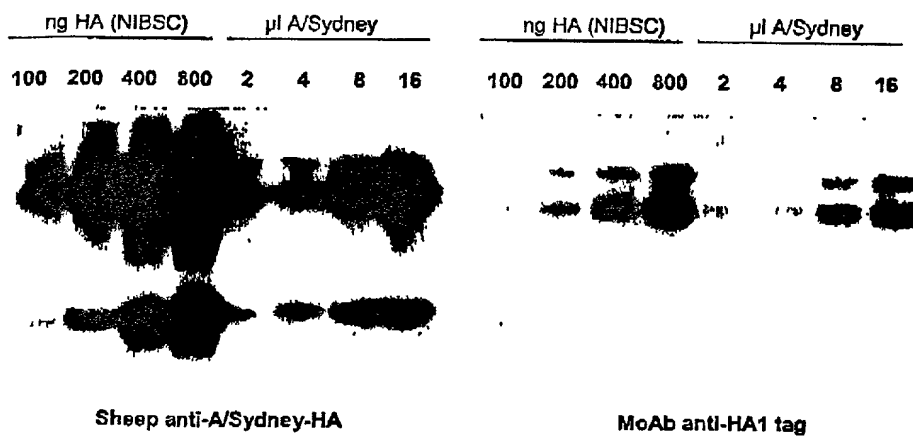


Fig. 17

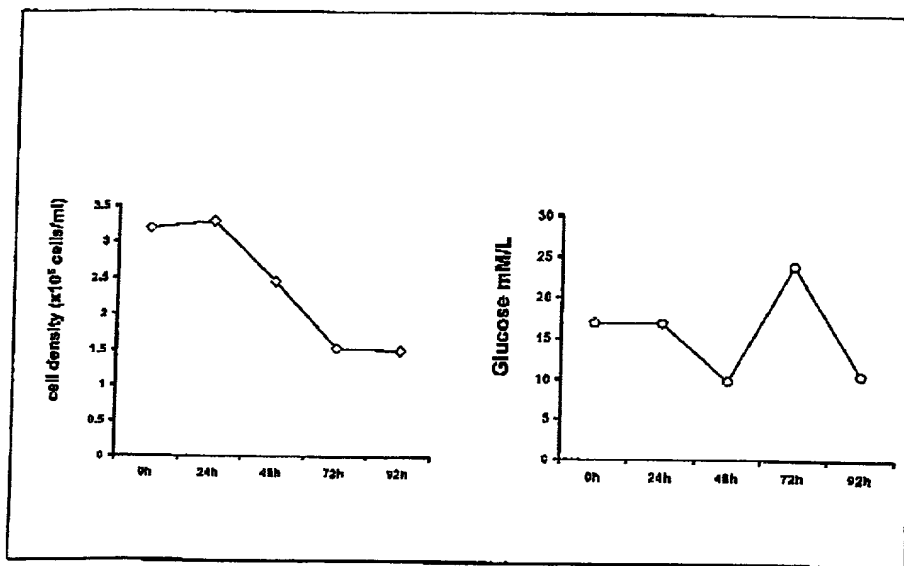
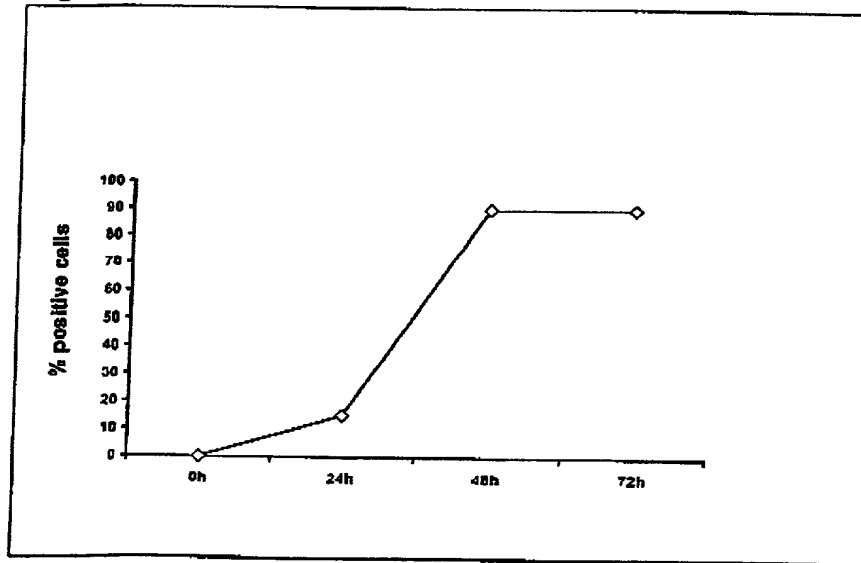


Fig.18





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Fig. 19

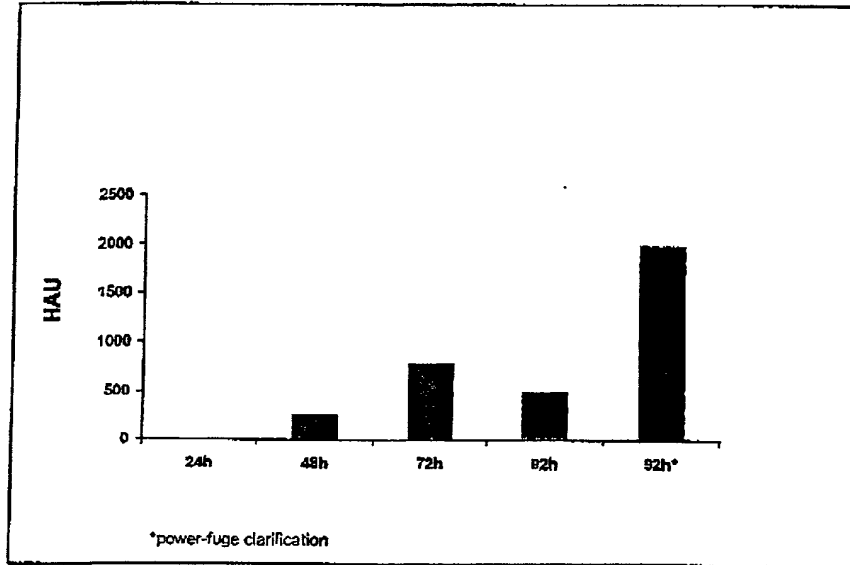


Fig.20

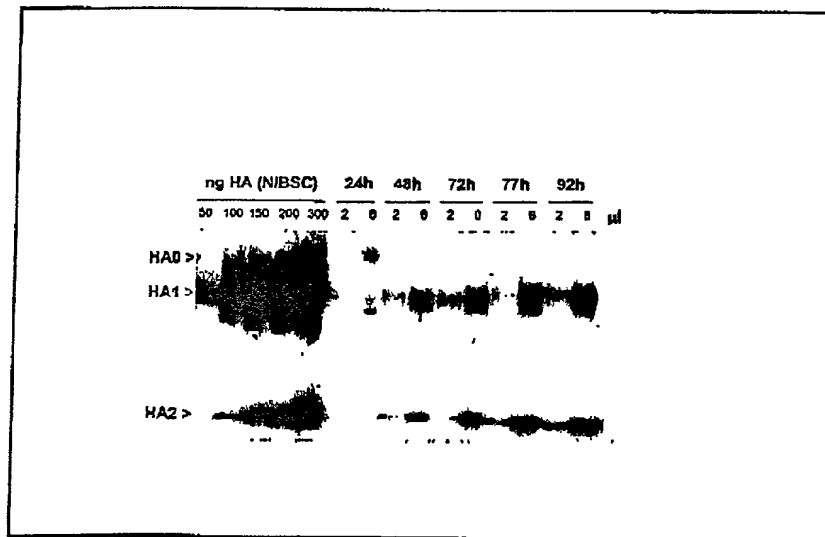


Fig. 21

	unconcentrated	20x concentrated
HAU	2048	98304
pfu/ml	1.80E+07	1.00E+08

00227 492269

Fig. 22

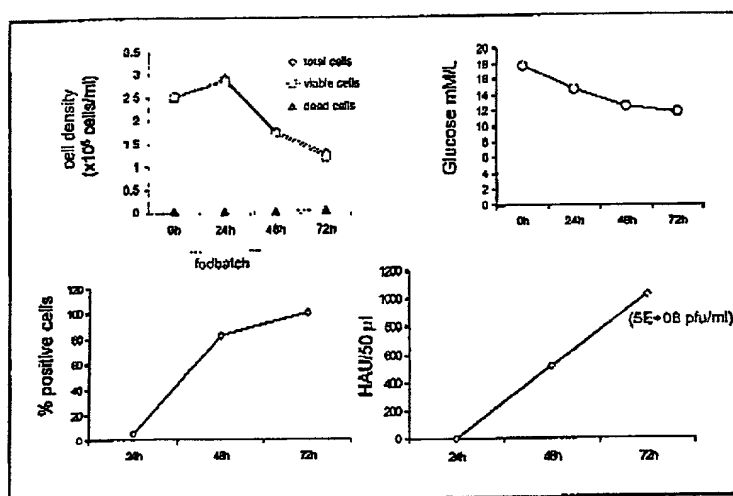


Fig. 23

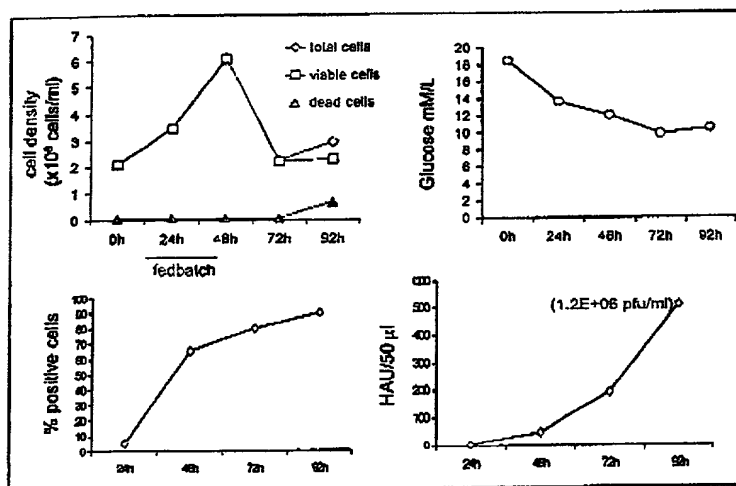


Fig.24

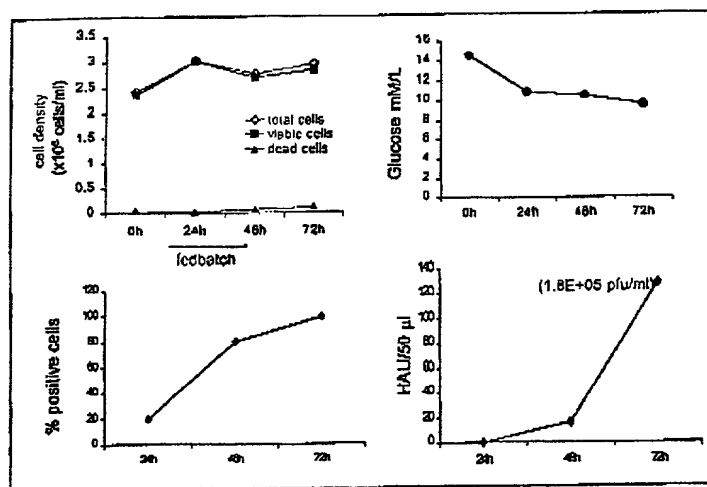


Fig 25

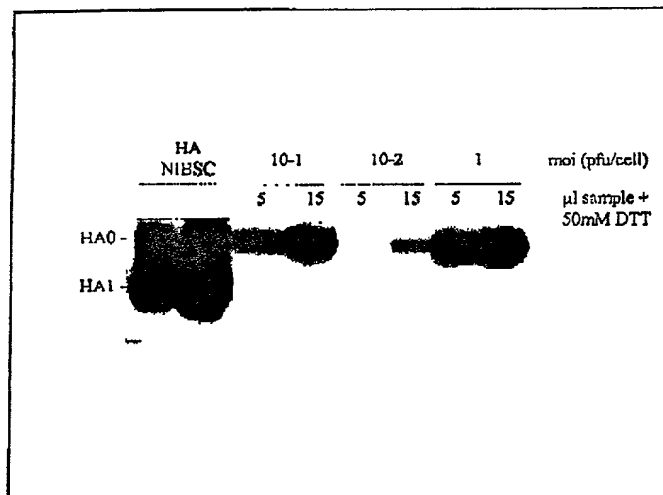


Fig. 26A

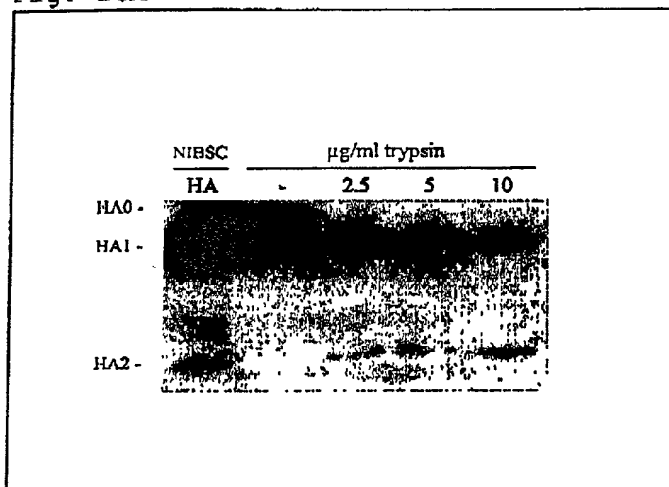


Fig. 26B

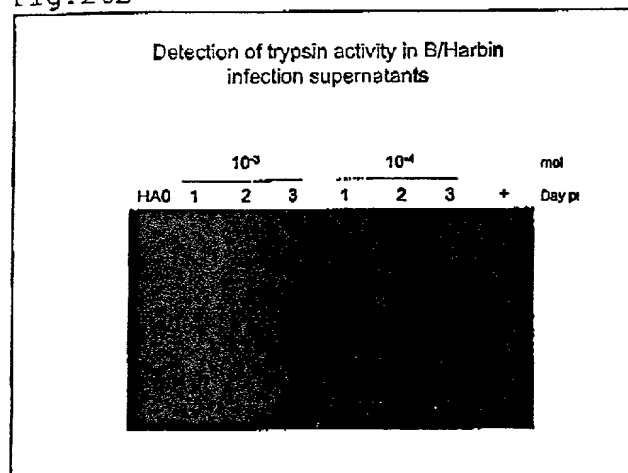


Fig 27

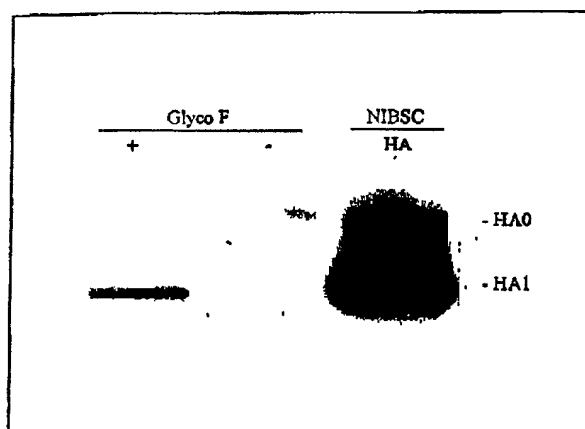


Fig 27

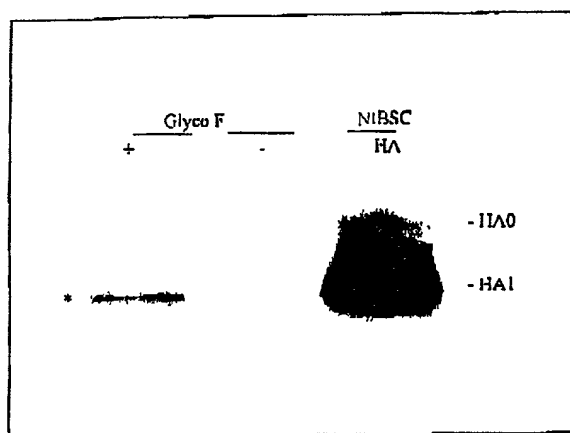
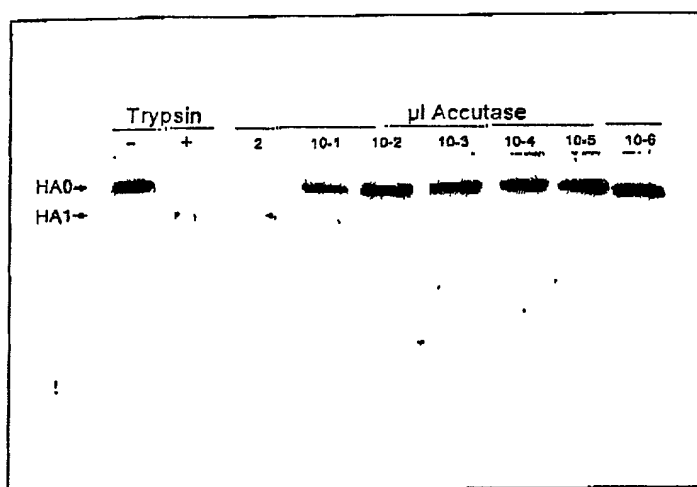
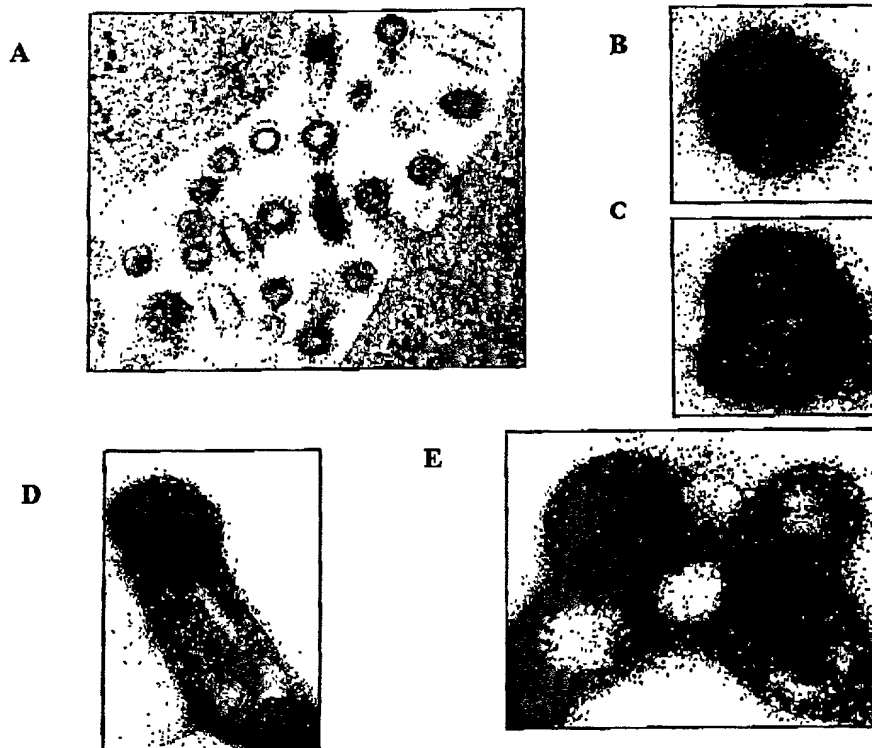


Fig.28



00221 112700

Fig. 29



09722867.112700



Fig. 30A

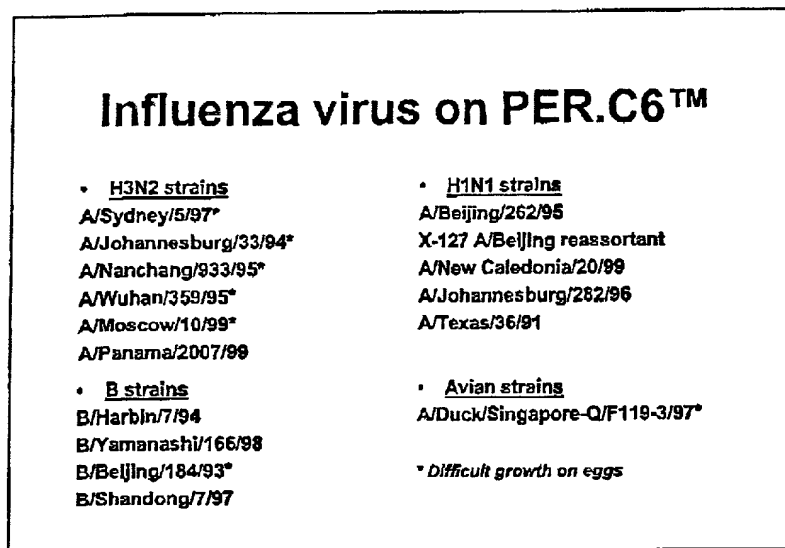


Fig. 30B

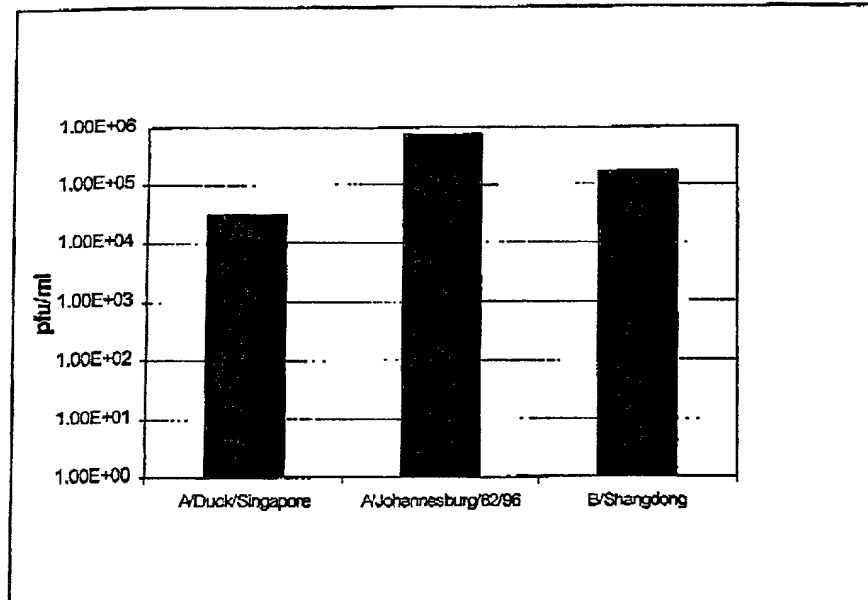
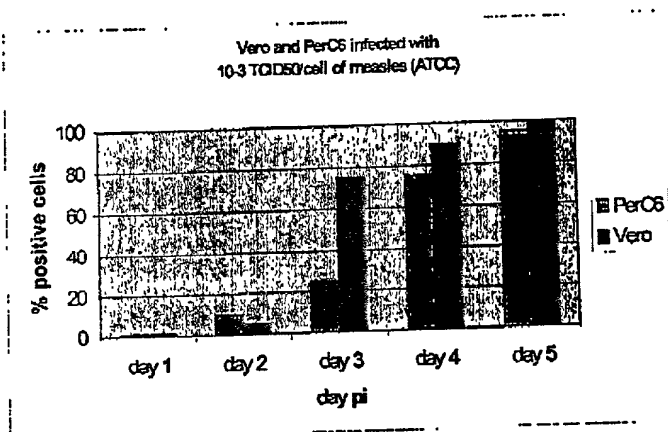
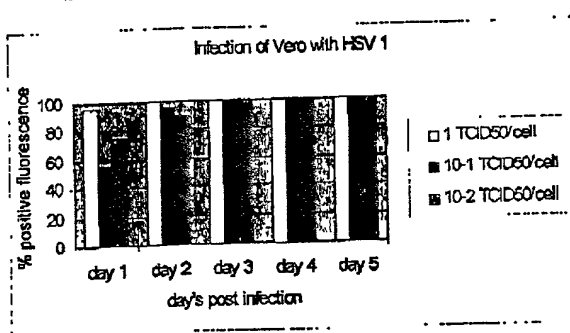


Fig. 31

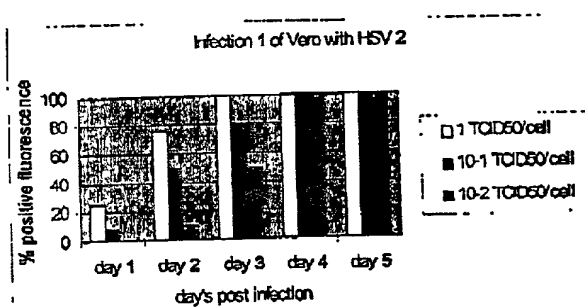
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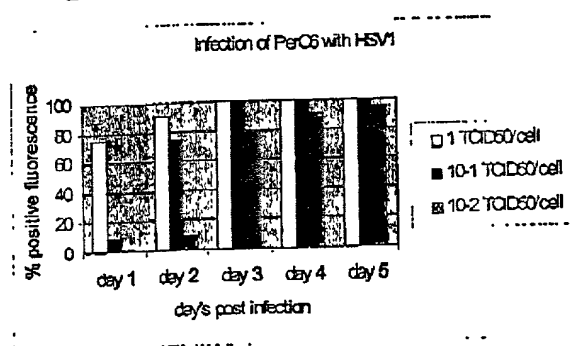
B



C



D



E

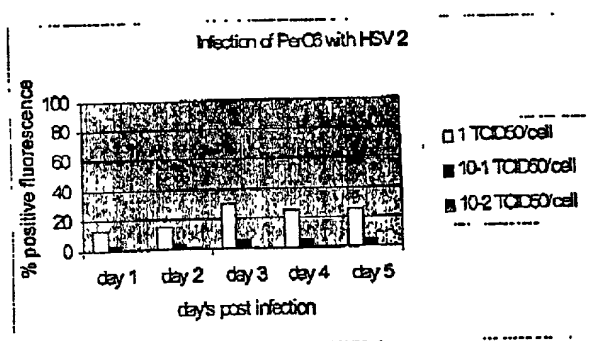


Fig. 32

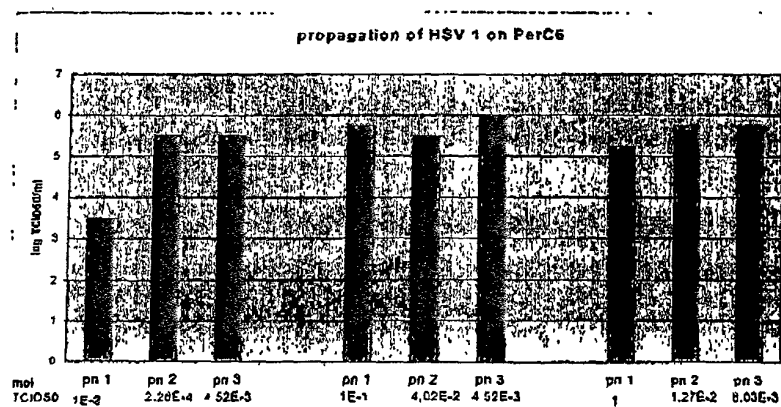
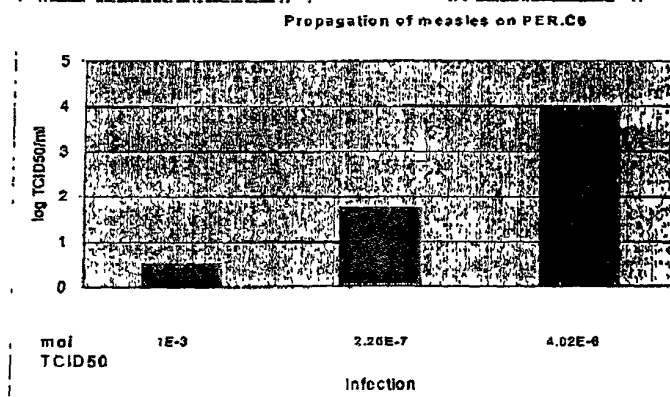
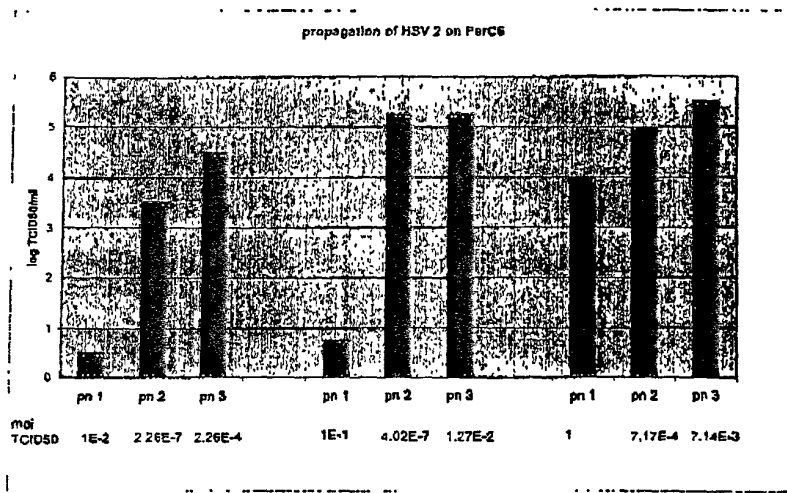


Fig. 33

